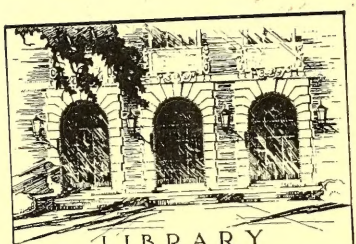

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# MORPHOLOGY AND DEVELOPMENT OF *NOSEMA NOTABILIS* KUDO

Parasitic in *Sphaerospora polymorpha* Davis,
A Parasite of *Opsanus tau* and *O. beta*

BY
RICHARD R. KUDO

Price $1.25

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1944

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# MORPHOLOGY AND DEVELOPMENT OF *NOSEMA NOTABILIS* KUDO

Parasitic in *Sphaerospora polymorpha* Davis,
A Parasite of *Opsanus tau* and *O. beta*

WITH 12 PLATES AND 7 TEXT FIGURES

BY

RICHARD R. KUDO

CONTRIBUTION FROM THE DEPARTMENT OF ZOOLOGY
OF THE UNIVERSITY OF ILLINOIS

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1944

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# I. INTRODUCTION

Of a number of cases of hyperparasitism reported in literature, in which a parasitic protozoan is parasitized by another protozoan, four microsporidians have been reported to occur as hyperparasites. Lutz and Splendore (1908) noticed the occurrence of *Nosema balantidii* in *Balantidium* sp., a ciliate, inhabiting the colon of *Bufo marinus* in Brazil. The spores of this microsporidian, which were found scattered, or in groups of four or eight, in the cytosome of the host ciliate, were pyriform and measured 2-5 $\mu$ by 1-3 $\mu$. Since these authors failed to prove the presence of the polar filament in the spore, the microsporidian nature of the organism is open to question.

Léger and Duboscq (1909) reported the occurrence of *Nosema frenzelinae* in the cytosome of *Frenzelina conformis,* a cephaline gregarine, which was parasitic in the intestine of a crab, *Pachygrapsus marmoratus,* at Cavaliere, France. The same two authors (1909a) found another microsporidian, *Perezia lankesteriae,* in the cytosome of *Lankesteria ascidiae,* an acephaline gregarine, parasitic in the intestine of a tunicate, *Ciona intestinalis,* at Cette, France. In 1919, Stempell observed *Nosema marionis* (Thélohan) in the cytosome of the trophozoites of *Ceratomyxa coris,* a myxosporidian, inhabiting the gall bladder of *Coris julis* and *C. giofredi* from French waters. This microsporidian had previously been noticed by Thélohan (1895) who, however, believed that the whole of the trophozoites were of a microsporidian, consequently, he considered the organism a coelozoic microsporidian, and named it *Glugea marionis.*

Recently I have observed the fifth case of microsporidian infection in a parasitic protozoan. In the summer of 1939, while examining the fishes of Chesapeake Bay for protozoan parasites at Solomons Island, Maryland, I discovered that the trophozoites of the very common myxosporidian *Sphaerospora polymorpha* Davis, inhabiting the urinary bladder of the toadfish, *Opsanus tau,* were frequently infected by a microsporidian for which the name *Nosema notabilis* was proposed (Kudo, 1939). It became known soon afterwards that this microsporidian attacks only the myxosporidian and does not infect any of the host fish cells. The infected myxosporidian trophozoites showed a cytological change characteristic of microsporidian infection. It therefore appeared certain that the microsporidian was a true and exclusive parasite of the myxosporidian. Since the microsporidian develops within small and isolated myxosporidian trophozoites, it seemed to present ideal material for me to renew work on this group of Protozoa in which I have been interested since 1910. In working out the morphology and development of *Nosema notabilis,* it became necessary to carry on a similar study of the host myxosporidian. For this purpose the toadfishes of Maryland and Florida were studied

here at Urbana, Illinois. Although the study did not reveal the complete life cycles of these two cnidosporidians, it brought to light certain important facts and new information which are here summarized.

Since this monograph was accepted for publication in December, 1941, data which appeared in papers after that time are not included.

## II. MATERIAL AND METHODS

The unique association of the two cnidosporidians under consideration was first discovered at Chesapeake Biological Laboratory, Solomons Island, Maryland, in July, 1939. During that summer the toadfish, *Opsanus tau,* were caught with hook and line or with seines in the vicinity of the Laboratory. During late fall and winter when the fish remained buried in mud, Mr. D. H. Wallace of the Laboratory used oyster tongues to good advantage. This confirms the earlier observations of Ayres (1842), Storer (1885), and others, as quoted by Gudger (1910), to the effect that the toadfish move to deeper water and bury themselves in the mud during the colder months. I am greatly indebted to Mr. Wallace for the collection and preservation of the material during October and December, 1939. In all, thirty-two fishes, varying from 10 to 25 cm in length, were studied from the Chesapeake Bay at Solomons Island.

During December, 1940, and January to March, 1941, fifty *Opsanus beta,*[1] 8 to 28 cm long, were obtained from Lemon Bay, Englewood, Florida. These fishes were received in shipping tanks on different dates. All arrived alive at Urbana, Illinois, except fifteen which apparently had died during the period of transportation. The living fishes were kept under observation in aquaria for up to three weeks from the day of their arrival.

For observation of living organisms, Nemeczek's (1926) hanging drop preparations, which I have been advocating for several years for the study of Myxosporidia, were made with the urine in which the organisms lived. Numerous cover-glass smears of various thickness were prepared by spreading drops of urine and scrapings of the bladder epithelium. The myxosporidian trophozoites are mostly attached to the bladder epithelium, so that smears that were made from empty bladders showed abundant and rich material. Many bladders, together with the ureters and portions of the kidney, were fixed in toto and serially sectioned 2-8 $\mu$ thick in paraffin.

As fixatives, Schaudinn's, Carnoy's, Bouin's, and Flemming's (strong) solutions were used as in previous works. Staining was carried on with Heidenhain's iron haematoxylin or Giemsa's solution in addition to

[1]Schultz and Reid (1937) consider that the toadfishes from the west coast of Florida are distinguishable from *O. tau.* I am indebted to Dr. C. L. Hubbs, of the University of Michigan, for information concerning these fishes.

Feulgen's nucleal reaction. The following combinations gave the best results: Carnoy, Schaudinn, Flemming, or Bouin and Heidenhain; Carnoy or Schaudinn and Giemsa or Feulgen.

The extrusion of the polar filaments of the spores of *Sphaerospora polymorpha* was easily accomplished as before by addition of potassium hydrate or hydrogen peroxide, or under mechanical pressure (Kudo, 1918, 1920a). The spores of *Nosema notabilis* were comparatively much fewer than in the other Microsporidia which I studied; for example, *Nosema bombycis* (Kudo, 1913, 1916) or various microsporidians of mosquito larvae (Kudo, 1921a, 1924, 1925, etc.). The methods employed successfully previously were unsatisfactory for observation under the dark-field microscope of the species under consideration. Hydrogen peroxide treatment of the spores brought about the filament extrusion in the usual manner, but it required a great deal of time to recover in dark field the affected spores in a highly agitated medium. However, by using a changeable condensor and apochromatic objective 20, it was possible to detect a few fresh spores in a very thin smear preparation in bright field. A dissecting needle with a bent tip was pressed down on the upper surface of the cover-glass while under observation, and when an optimum amount of mechanical pressure was thus applied, the polar filaments were extruded from the spores, which now became less refractile and more rounded. The extruded polar filaments can hardly be recognized in bright field (fig. 167), but are easily observed by quickly changing the field from bright to dark. The same manipulation was carried on equally successfully with an apochromatic oil immersion objective 60 with a built-in diaphragm, in which case the objective was raised at the time of the application of the needle to the cover-glass (figs. 166, 167). For permanent preparations of the spores of *Nosema notabilis* with their extruded filaments, I obtained satisfactory results by using the methods previously employed (Kudo, 1913, 1918, 1921).

## III. URINARY SYSTEM OF *OPSANUS TAU* AND *O. BETA*

Since the two cnidosporidians with which the present paper deals have so far been found only in the urinary bladder of *Opsanus tau* and *O. beta*, it was necessary to obtain fuller information than that available in literature on the anatomy of the system in these host fishes. Comparative examinations indicate that the system in the two species of toadfish possesses the same morphological characteristics.

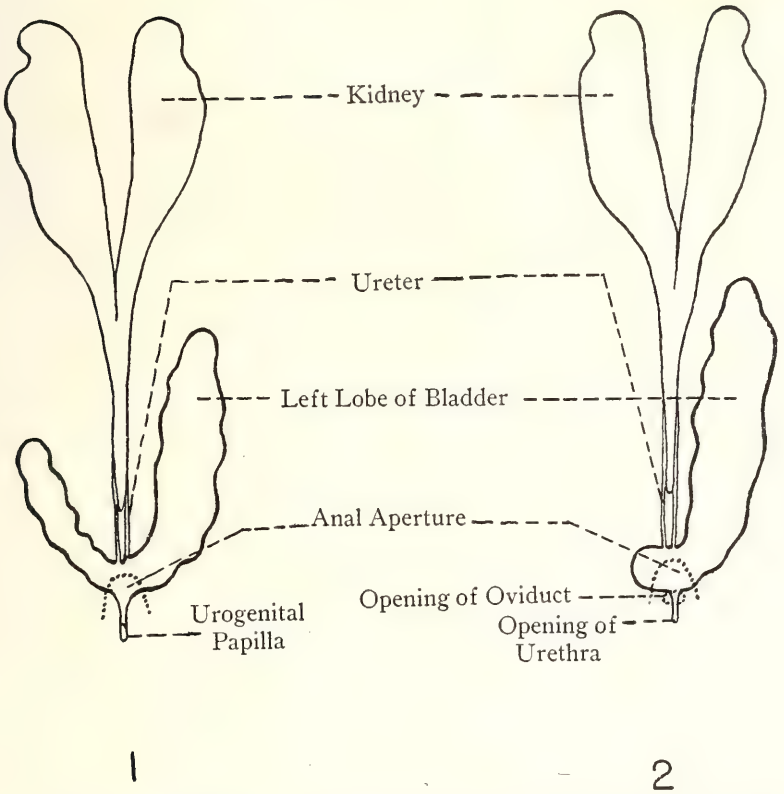
The relatively large compact kidney is a paired organ, extending over a large area on the dorsal surface of the body cavity. The anterior two-

fifths are separated into the right and left lobe, while the remaining portion is fused into a slender median band which tapers to a fine strand at its posterior end. In a fish 10 cm long, the kidney was about 3 cm long, the anterior region of the two lobes 4-6 mm wide, and the posterior fused part 13-15 mm long by 1-3 mm wide. During the course of the study, serial sections of the kidneys of several fishes were carefully examined with expectation that certain stages in the development of the two cnidosporidians might occur in the kidney. The search ended in disappointment, but it revealed that the toadfish kidney contains no glomeruli, which condition had first been discovered by Marshall (1929).

From the dorso-lateral edges of the posterior portion of the kidney arise two ureters, which extend side by side along the median line of the body and open into the urinary bladder. The ureters vary from 5 to 10 mm in length in fishes 10 to 20 cm long, as measured between the posterior tip of the kidney and the points of attachment to the urinary bladder. The two ureters open independently into the bladder. In a female fish 12 cm long, which had been fixed with Carnoy's fluid and sectioned, the distance between the openings of the two ureters was 450 $\mu$ as seen in serial sections.

The urinary bladder is situated above the gonads, and, except in the posterior region, is without any tissue attaching it to the peritoneal membrane or viscera, being freely suspended in the body cavity. There is a sexual dimorphism with respect to the form and arrangement of the bladder. In the male fish, there are right and left horns or lobes which are connected with each other posteriorly (text fig. 1). In all fishes examined, the left horn contained far greater amounts of fluid than the right one. In a number of fishes, the left horn was fully distended and reached the anterior end of the body cavity, while the right horn appeared empty, narrow, and small, with a wrinkled wall. At first it was thought that prior to dissection, the right horn may have emptied its contents, which would result in its smaller size. But when fishes which have been dead a short time are opened, both horns of the bladder are usually not inflated, and here again the difference in dimensions between the larger left and smaller right horns is conspicuously noticeable. It appears certain, therefore, that the left horn is naturally much larger than the right one. The anterior region of the left horn in particular, appears occasionally to show a tendency to remain distended after the remaining part emptied its contents, due possibly to strong transverse muscles located in the bladder wall.

In a freshly sacrificed male fish, 13 cm long, the left horn was expanded and measured 3 cm long by 9 mm wide at the broadest point, while the right horn was 12 mm long by 4 mm wide. In another male fish, 22 cm long, examined about 48 hours after death, both horns were contracted. The left horn measured 28 mm long by 3 mm wide, and the




TEXT FIGS. 1-2.—Diagrams of the urinary system of the two species of Opsanus, as seen from the ventral side; 1, male; 2, female.

right, 17 mm long by 1.5 mm wide. In a third male fish, 10.5 cm long, examined after death, the left horn was 13 mm long by 2 mm wide near its posterior end, while the right horn was 4 mm long by 1 mm wide.

In the female fish, the left horn is always conspicuously noticeable, but the right horn does not occur (text fig. 2). The left horn is similar in form and appearance to that of the male fish. In two female fishes, both 12 cm long, which died during transportation, the urinary bladder measured 20 mm long by 3 mm wide and 15 mm long by 4 mm wide. In a freshly sacrificed female fish, 15 cm long, the highly distended bladder, located above the left ovary, measured 33 mm long by 8 mm wide.

The urethra is a short tubule connecting the posterior end of the bladder with the urinary pore, which is located behind the opening of the oviduct and in turn opens posterior to the anal aperture. The urine or fluid content of the bladder is colorless and translucent. Its pH as measured by the calorimetric method averages 7.2.

These findings will now be compared with those of previous observers. The first observation on the urinary organ of the toadfish appears to have been made by Hyrtl (1851). Studying a female *Batrachus tau,* which was "5 Zoll" (about 12.7 cm) long, Hyrtl made the following observation: "Die Blase selbst liegt links vom linken Ovarium, hat 9 Linien Länge, und besteht aus einem hinteren 3 Linien weiten, und einem vorderen nur 2 Linien weiten Abschnitte. Die anderthalb Linien lange und feine Urethra mündet auf der hinteren Lefze der weiten Genitalöffnung." Hyrtl gave a figure (his pl. 14, fig. 3) which is reproduced here in text fig. 4. Thus the single-lobed condition of the urinary bladder of the female toadfish had correctly been observed by Hyrtl.

3 4

TEXT FIGS. 3-4.—Hyrtl's illustrations; 3, the urinary bladder of *Batrachus cryptocentrus:* a, "left flap of bladder," b, "diverticulum on its crown," c, "right, narrower and shorter flap"; 4, the urinary bladder of *Batrachus tau,* "composed of two unequally wide segments."

The same author examined a male *Batrachus cryptocentrus,* "6 Zoll" (about 15.2 cm) long, and described his findings as follows: "Zwei Ureteren treten in den Rücken einer asymmetrisch zweilappigen Blase, deren rechter Lappen schmäler und etwas langer als der linke ist. Beide Lappen sind sehr ansehnlich, und liegen an den entsprechenden Bauchwänden an. Der Linke ist noch überdiess an seinen gerundeten Scheitel mit einem 1½ Linien langen, engen Diverticulum versehen. Es findet sich nur eine einfache Urogenitalöffnung auf einer niedrigen und dünnen Papille am hinteren Afterrande." Hyrtl gave a figure (his pl. 14, fig. 2), which is reproduced here as text fig. 3. This figure does not bear out what he stated in the text, since in his explanation of this figure, Hyrtl stated that *a* indicated "linker Lappen der Blase," *b,* "Diverticulum an dessen Blase," and *c,* "rechter, schmälerer und kürzerer Scheitel." Thus his text statement based on one male fish is in discrepancy with the illustration, presumably taken from the same fish. If he erred in the text and was correct in the illustration concerning the right and left sides, my observation of the urinary system of the male Opsanus is in accord with that of the male *Batrachus cryptocentrus* as observed by Hyrtl.

Gudger (1910), in his studies on the habits and life history of the toadfish, gives photographs of the gonads and urinary bladder of *Opsanus tau* which are unfortunately indistinct in the copies I have examined. His fig. 1-A, a dorsal view of the male organs, appears to show the left horn of the bladder as longer and wider than the right. The sperm duct and urethra open independently according to Gudger, as he writes: "They [elongated glands] are confluent behind to form the sperm duct, which opens in the same place and manner as the oviduct." In the description of the ovary, Gudger stated that "in fig. 1 the anus is shown and to the left of it one of the paired halves of the urinary bladder." This leads me to think that Gudger observed paired lobes in the bladder of a female toadfish. His fig. 2, labelled "ventral aspect of living ovary," appears to be in reality a dorsal view, because the urinary bladder appears above the (left) ovary, and also judging from the erroneously placed label for fig. 1-C in which the anal aperture is visible. If this interpretation of Gudger's photographs is correct, there appears to be a single sac for the urinary bladder located close to the ovary in his photograph, and his observation agrees with my observation mentioned above.

Marshall (1929), who made a careful histological study of the kidney, makes the following statement: "The bladder is bi-lobed but the lobe on the right is usually very small in comparison with that on the left. The two lobes are in communication. The ureters pass up from the bladder on each lateral edge of the fused posterior portion of the kidney, and then disappear into the substance of the middle portion of the organ." He does not mention any difference in the form of the bladder between the male and female fishes. However, referring to Hyrtl's work quoted above, Marshall states that "his description of *Batrachus cryptocentrus* is evidently that of our toadfish rather than his account of *Batrachus tau;*" since the latter was a female fish, I am led to assume that Marshall did not recognize the sexual dimorphism in the urinary bladder of the toadfish *Opsanus tau.*

Because of its easy access and hardy nature, the toadfish has occasionally been used by physiologists for investigations on osmotic regulation, urine flow, diuresis, etc. For example, Graffin (1931) used *Opsanus tau* for his study on urine flow and diuresis. The fishes were collected in the vicinity of Baltimore, a short distance from Solomons Island where all toadfishes were found to be parasitized more or less heavily by *Sphaerospora polymorpha,* which in turn was infected to a varying degree by *Nosema notabilis.* It is assumed that the two cnidosporidians consume substances present in the urine, as well as in the bladder epithelium, for growth and reproduction. There is no way of estimating the amount of substances consumed by them and how much catabolic waste matter is excreted by them, but in a heavy infection, in which the bladder epithelium is completely covered by one to many layers of trophozoites of the

myxosporidian, the amount may be considered relatively large. The urine analysis of the toadfish, therefore, indicates in reality the sum-total of the excretion by the host fish into the bladder and of the catabolic products excreted by the two protozoans, less the material used by the latter organisms, and it obviously does not give the true picture of the urinary excretion of the toadfish itself.

## IV. *SPHAEROSPORA POLYMORPHA* DAVIS

### Occurrence

As was stated already, eighty-two toadfishes, varying in length from 8 to 30 cm, were examined during July, August, November, and December, 1939, December, 1940, and January to March, 1941. All the fishes harbored the myxosporidian in the urinary bladder in varying numbers. Even when the trophozoites or the spores could not be detected in fresh preparations, a small number of trophozoites attached to the bladder epithelium could be observed in section preparations. In the bladder of the majority of the fishes examined, the myxosporidian was abundantly present. Although numerous trophozoites were seen floating freely in the urine of the bladder (fig. 159), the majority were attached to the bladder epithelium; in fact, the inner surface of the bladder wall was completely or in part covered by trophozoites, which were arranged in so compact a fashion in a single layer that the organisms showed all possible angular forms (text fig. 5). In several fishes, the bladder wall was covered in places by several layers of trophozoites of various sizes of the myxosporidian (fig. 162). The present study reveals that this myxosporidian is represented by all stages throughout the year, except in spring in which no examination has so far been made.

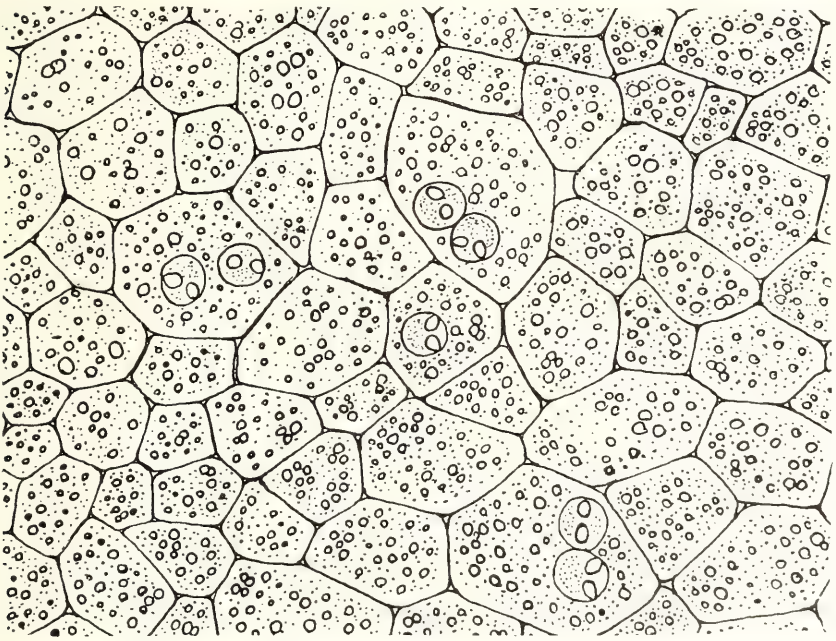
Davis (1917), who discovered and described this myxosporidian, examined 11 *Opsanus tau* at Beaufort, North Carolina, in June and July, and found 9 harbored the organism abundantly. He stated: "The trophozoites are usually attached to the urinary epithelium which in badly infected fish may be almost entirely covered with them."

Of numerous Myxosporidia that are parasitic in the urinary bladder of various fishes (Kudo, 1920a), *Sphaerospora elegans* and *S. divergens* were found in both the renal tubules of the kidney and the bladder. In the case of *Myxidium lieberkühni,* the well-known species inhabiting the urinary bladder of species of Esox in North American and European waters, Debaisieux (1918) found that it invaded not only the bladder, but also the uriniferous tubules and even the glomeruli of the kidney, in which the organism produced cysts. *Sinuolinea dimorpha* was found by Davis (1916, 1917) in the urinary bladder and Wolffian ducts of *Cynoscion*

*regalis.* On the other hand, there appear to be a number of myxosporidians which inhabit the bladder only. For example, Auerbach (1909) found *Zschokkella hildae* only in the bladder of *Phycis blennioides, Gadus callarias,* and *G. virens* at Bergen, Norway.

*Sphaerospora polymorpha* appears to belong to the latter group in that it inhabits or passes its major portion of existence in the urinary bladder of the toadfish only. Repeated search in serial sections of the ureters and kidneys of several host fishes, the bladder of which showed a heavy infection by this myxosporidian, failed to reveal any organisms in the lumina of ureters or uriniferous tubules, lymphoid tissue cells, or blood vessels of the kidneys. In the wall of the ureters of a single host fish examined in fresh condition, a small number of coccidian oocysts of apparently one kind, but in various stages of development, were observed. The mature oocyst contained four spores, each with two sporozoites, which character places the coccidian in the genus Eimeria.

Linton (1901, 1905) showed that the toadfish at Woods Hole, Massachusetts, and Beaufort, North Carolina, were hosts to numerous helminth parasites. The same has been true with the fishes from Maryland and



TEXT FIG. 5.—Surface view of portion of the inner surface of the urinary bladder of a toadfish completely covered by the trophozoites of *Sphaerospora polymorpha.* In life, somewhat flattened. × 1060.

Florida which I have examined during the present study. Every toadfish was infected by several individuals of species of helminths, in tissues and organs associated with the reproductive and urinary systems. Certain of these worms were examined in life, as well as in section preparations, but none harbored either of the two protozoans discussed here.

As to the possible effect of the infection on the host fish, nothing definite can be said at the present moment, since no host fish was entirely free from infection. The behavior and activity of the host fish under laboratory conditions did not indicate the intensity of infection of the bladder by the myxosporidian. Nor were there any external symptoms to show a heavy infection.

Smaller trophozoites appear to be lightly attached to the inner surface of the bladder epithelium, and, therefore, do not seem to affect the cell contents of the tissue cells. As they grow, the attachment becomes firmer, and the trophozoites appear to destroy the cytosome of the host cells, which become increasingly smaller; the trophozoites finally come to lie in the depression of the epithelial cells (fig. 161, center left).

In the bladders studied in serial sections, the thickness of the epithelium or the length of the component epithelial cells varies a great deal. The trophozoites are usually seen attached to the smaller epithelial cells, while the columnar cells adjacent to them do not show any attached organism (fig. 161). It was thought at first that this was due to destruction of columnar epithelial cells by the myxosporidian. However, it was found that this was not the case, for in lightly infected fishes, thin and thick epithelial cells occur without the influence of the parasitic organisms. It may be assumed that the columnar epithelial cells are actively secretional in function, and constantly produce secretions which would make the attachment of trophozoites impossible.

The attached trophozoites grow obviously at the expense of the substances of the host cells to which they are attached, as well as of the liquid and solid substances present in the urine which surrounds them. The epithelial cell nuclei, which are situated near the bases of the cells, do not show any noticeable change as a result of destruction of the outer halves of cells as observed in numerous section preparations treated with Feulgen's nucleal reaction or stained with the nuclear dyes. Thus my observation on the effect of the infection upon the host tissue is in agreement with that of Davis (1917), who stated: "The young trophozoites are attached to the free ends of the epithelial cells (fig. 93) which, however, show no signs of injury. As the trophozoites increase in size they come to lie in depressions formed by the destruction of the ends of epithelial cells (fig. 94). Often the injury to the epithelial cells is carried much further than shown in the figure, in some cases the part of the cell

immediately surrounding the nucleus being all that is left, but even in such cases the nucleus shows little sign of injury."

Generally speaking, unlike histozoic Myxosporidia, coelozoic forms seldom bring about injury to the host organs in which they live. There are, on the other hand, a certain number of authors who observed pathological changes caused by coelozoic myxosporidians on the host organs, such as the gall bladder, urinary bladder, etc. For example, Bauer (1921) reported a cystitis of the bladder of *Esox lucius* infected heavily by *Myxidium lieberkühni.* Even in the most heavily infected bladder no hypertrophy or degeneration of the nuclei of the host epithelium has been noticed in the present myxosporidian. Nor was there any indication that "toxic" stimulation is exercised by the parasite upon the host organ, as was suggested by the same author.

## The Trophozoite

In his original diagnosis of the trophozoite of *Sphaerospora polymorpha,* Davis (1917) gave the following description: "Colorless, usually somewhat elongate (fig. 90), but never very irregular in shape; slowly amoeboid. After being on the slide for a short time become rounded and motionless (figs. 86-89). Ectoplasm distinct around younger trophozoites, hyaline, forming one to several large lobate pseudopodia, which in turn extrude several short, conical pseudopodia (fig. 88). In larger trophozoites ectoplasm often not distinguishable except at ends of pseudopodia, which in such cases are composed chiefly of endoplasm. Endoplasm distinctly granular, sometimes vacuolated in smaller trophozoites (fig. 88), but in larger individuals vacuoles are indistinct or absent; small fat globules usually abundant, especially in larger individuals; numbers of rounded sporoblast cells can be distinctly seen."

The majority of the trophozoites are attached more or less firmly to the epithelial cells of the bladder, although many others are suspended freely in the urine. Extremely small trophozoites (fig. 1), measuring less than 10 $\mu$ in diameter, are difficult to identify as such in life, except when infected by *Nosema notabilis* (figs. 8, 9), but larger individuals, with their diameters 10 $\mu$ or more, can be distinguished without difficulty. As examined in Nemeczek's preparations, the trophozoites that are free in the urine are more or less spherical or rounded (figs. 1-3), or may assume somewhat elongated forms when undergoing change of body form (figs. 4-6). The body form change is, as a rule, carried on very slowly, but in certain individuals is quite rapid. The pseudopodia are ordinarily produced from the comparatively limited area which is in contact with the bladder epithelium, although in some individuals, there may be numerous pseudopodia distributed over the entire body surface (fig. 6). The

pseudopodia are lobose (figs. 2, 3, 12) before they are fully formed, usually broad at the bases and terminating in one or more conical secondary pseudopodia which are sharply attenuated (figs. 4-7), as is the case with numerous coelozoic myxosporidians (Kudo, 1920a).

After being on the slide in the urine of the host fish, the trophozoites become inactive, rounded, and sooner or later degenerate. If the room temperature remains below 20° C., some of them still show noticeable changes of body form after 16 to 20 hours. They appear to retain vitality much longer if they remain in the bladder, even long after the death of the host fish. In one instance, a male toadfish which was dead when it arrived was kept in a refrigerator at about 4° C. for two more days before dissection and examination. More than 50 per cent of trophozoites of various sizes were perfectly normal in appearance, and numerous individuals showed active formation of pseudopodia. Thus, in the case of *Sphaerospora polymorpha,* the trophozoites are able to live in the host organ for at least 50 hours after the death of the host fish, in contrast to the belief sometimes expressed that the active life of a parasitic protozoan ceases with the death of its host.

Trophozoites with a diameter of 10-25 $\mu$ are the most abundant in host fishes examined up to the present time (text fig. 5; fig. 162). Larger trophozoites occur less frequently. They are elongated and leaf-like, ellipsoidal, club-shaped, or moniliform in outline. They may reach 100 $\mu$ in length, but the body is usually slender. The majority of larger forms are 50-80 $\mu$ long by 20-40 $\mu$ wide (figs. 15, 16, 161). Davis (1917) wrote that large vegetative trophozoites were about 35 by 50 $\mu$.

The cytoplasm of the trophozoite is colorless and hyaline, and is differentiated into the ectoplasm and endoplasm. The ectoplasm is distinct in the peripheral zone, as well as in the pseudopodia, and does not contain any inclusions. The endoplasm, which makes up the bulk of the body, is variously vacuolated and contains highly refractile granules of variable dimensions, less refractile bodies, granules, small crystals, and other cell inclusions. The appearance of the trophozoites is very similar to other coelozoic myxosporidians I have studied, especially the small trophozoites of *Myxidium lieberkühni* (Kudo, 1921b). The highly refractile spherules, which vary in diameter from about 0.2 to 2.5 $\mu$, dissolve in alcohol, but not in dilute acetic acid or water. Sudan III stains them intensely red (fig. 10). They thus appear to be fat globules.

The less refractile ill-defined bodies, which often contain several minute granules, are stained green against violet-bluish cytoplasm when living trophozoites are treated with acidified methyl-green and are without doubt the trophozoite nuclei. The crystals are regularly rhombic plates and were found in a small number of trophozoites occurring in a few

fish in which they were abundantly present in the epithelial cells of renal tubules of kidney, ureters, and bladder. They may be similar to the haematoidin crystals reported in *Myxidium lieberkühni* (Bütschli, 1881), *Sinuolinea dimorpha* (Davis, 1917), etc. As the central area of the body becomes stained reddish-brown after iodine treatment, there may occur diffused glycogenous substance also. In the trophozoites, which are more or less cylindrical, the part that is in close contact with the epithelial cells appears to be denser and stains more deeply with a cytoplasmic stain than other part of the body, which condition was previously noted by Davis (1917).

The trophozoites are seemingly able to engulf solid particles that are present in the urine. These particles are crystals, spheroidal bodies which are yellowish to brownish and measure up to about 5 $\mu$ in diameter (fig. 16), and rounded homogeneous bodies measuring 5-20 $\mu$ in diameter (figs. 28, 29). The latter take stains homogeneously and do not reveal any structures which react positively to Feulgen's nucleal reaction (fig. 29). When seen in life, they were looked upon as gemmules that have been observed quite frequently in *Leptotheca ohlmacheri* (Kudo, 1922). Because of their structurelessness and of the presence of similar bodies in the bladder epithelium, I think that these conspicuous homogeneous bodies are excretion products of the bladder epithelium which were engulfed by the trophozoites.

Occasionally the trophozoites are seen ingesting free spores (fig. 13) or other solid material present in the urine. Ingestion of solid particles by myxosporidian trophozoites appears to have first been seen by Bütschli (1881) in *Myxidium lieberkühni,* who wrote: "Man sieht nämlich solche kleinen Formen häufig an losgelösten Epithelzellen der Harnblase derart befestigt, dass ein Theil der Zelle von der Myxosporidie umfasst und eingehüllt wird (fig. 30)." In recent years, Davis (1916) noticed ingestion and digestion of erythrocytes by *Sinuolinea dimorpha* while within the bladder of the host fish.

The trophozoites appear to undergo plasmotomy in the host's bladder, since in a number of individuals observed in life for several hours, plasmotomic division was noted (figs. 17, 18). Under an oil immersion objective, these individuals were found to contain several nuclei. In addition to the simple plasmotomy, multiple division probably occurs also. Larger trophozoites are usually elongated, and the body shows 3 to 5 swellings, between which the cytosome is extremely thin (fig. 15). They represent, most probably, a stage in multiple plasmotomy. Furthermore, the occurrence of enormous numbers of comparatively small trophozoites in the bladders of many host fishes and of closely associated groups of small trophozoites, as frequently seen in fresh preparations (fig. 11), points to multiple

plasmotomic division in the present myxosporidian. On the other hand, there is no indication of gemmation such as has been observed in *Leptotheca ohlmacheri* (Kudo, 1922). Plasmotomy has been observed in a number of coelozoic myxosporidians. Seeing a simple division of the trophozoite of *Chloromyxum leydigi,* Doflein (1898) called the process plasmotomy. This multiplication in *Myxidium lieberkühni* was observed by Cohn (1895), Laveran and Mesnil (1902), Bremer (1922), and others. Laveran and Mesnil observed simple plasmotomy of young trophozoites, while Bremer reported that the trophozoites which he examined multiplied by simple and multiple plasmotomy, in addition to endogenous budding.

Whether the spore germinates in the urine of the bladder in which it has been formed is not definitely known. A spore of *Myxidium lieberkühni* was seen by Bremer (1922) to germinate in the urinary bladder of a dead pike. Judging from the observations on autoinfection by different species of histozoic Myxosporidia (Kudo, 1926), its occurrence in coelozoic forms appears probable. In many smears, as well as section preparations, however, no germinating spores or empty spores have been noticed in the present species up to the present time.

### Development of Trophozoites and Process of Spore-Formation

Although, as was pointed out before, the nuclei of the trophozoite are recognizable in life, the process of division and changes which result in formation of spores could not be followed and studied in life, due to technical difficulties. As in the case of previous investigators of Myxosporidia, my observations are based on fixed and stained smears and sections of naturally infected urinary bladders of many host fish.

The smallest trophozoites observed in smears are shown in figs. 20 to 22. They are spheroidal or ovoid bodies about 5-9 $\mu$ in diameter. The cytosome is reticulated and stains light violet or bluish in Giemsa's solution. In the majority of these trophozoites, two nuclei were present, but in some there occurred only a single nucleus. In the latter form (fig. 20), the nucleus is lodged in a small mass of differentiated cytoplasm which stains blue, in contrast to the general cytoplasmic background. It measures about 2 $\mu$ in diameter, with peripheral chromatin granules and an endosome which is clearly achromatinic in nature. Compared with the sporoplasm of the mature spore (figs. 89-91, 94, 95), this uninucleate trophozoite differs in size and in having a mass of perinuclear cytoplasm. The difference in size, however, is not so important as the difference in cytoplasmic structure, since the sporoplasm of a large spore is comparable in size (figs. 96, 100), and, moreover, being in a thin portion of the smear, the body appears to be more or less flattened. In all well-studied Myxosporidia, the two sporoplasm nuclei fuse before or after germination from

the spore. The fused sporoplasm nucleus (fig. 90) is much smaller and more compact than that of the uninucleate trophozoite under consideration (fig. 20), and it is lodged in a homogeneous cytoplasmic mass, instead of in a differentiated cytoplasmic mass as in the latter form. Yet this uninucleate condition leads me to maintain that the trophozoite shown in fig. 20 is the youngest stage, which is the closest to the intrasporal sporoplasm.

The binucleate trophozoites shown in figs. 21 and 22 approximate in size the sporoplasm of average spores. There is not much difference in size between them and the sporoplasm which was apparently accidentally extruded from a developing spore by mechanical pressure during the process of preparation (fig. 86). However, the two nuclei in the accidentally extruded sporoplasm are much smaller and characterized by a thick peripheral chromatin zone, and are presumably destined to undergo fusion to produce the single nucleus of the sporoplasm (fig. 90). On the other hand, the two nuclei seen in the binucleate trophozoites (figs. 21, 22) are quite different in structure. One nucleus is similar to that in the uninucleate trophozoite (fig. 20), but the other is imbedded in the general cytoplasm and does not contain any achromatinic endosome. Later changes indicate that the nucleus with an achromatinic endosome and surrounded by a differentiated mass of cytoplasm is the generative nucleus; while the other, without such an endosome, is the vegetative or somatic nucleus.

Nuclear division of the uninucleate trophozoite has not been observed, but it seems probable that the nucleus divides and transforms itself into the two kinds of nuclei found in the binucleate trophozoite, as has been reported in *Sinuolinea dimorpha* (Davis, 1916), *Leptotheca ohlmacheri* (Kudo, 1922), etc.

While in the binucleate state, the trophozoites grow in size, the generative nucleus divides by mitosis (figs. 23, 28), and trophozoites with two generative nuclei and one vegetative nucleus (figs. 24, 25, 31) are formed. The vegetative nucleus has not been seen undergoing mitotic division, and appears to divide amitotically (fig. 26), as in the case of *Leptotheca ohlmacheri* (Kudo, 1922) and of several histozoic Myxosporidia (Davis, 1923; Debaisieux, 1924, 1925; Kudo, 1926). With the growth of body, the size and number of nuclei also increase. There is no definite ratio as to the numbers of the generative and vegetative nuclei. In many individuals (fig. 26), there are two generative and two vegetative nuclei; in some (fig. 27), two generative and four vegetative nuclei; and in still others (fig. 28), four generative nuclei and a single vegetative nucleus. In the sporulating individual shown in figs. 30 and 31, eight generative and five vegetative nuclei and two developing spores were noticeable.

Prior to division the generative nucleus becomes much enlarged, and its chromatin substance, which responds positively by intense coloration

to Feulgen's nucleal reaction, is distributed along the inner surface of the nuclear membrane as a thin but distinct zone. Attached to this zone are chromatin granules which are connected with one another by chromatinic threads (fig. 32). The whole chromatin material then becomes transformed into a spireme (figs. 23, 33). The spireme shortens and breaks up into four chromosomes (figs. 34, 35) which become arranged at equatorial plane (figs. 28, 36-38, 156). At this stage, the chromosomes become short and compact.

The achromatinic fibers make their appearance about the time the chromosomes become distinctive (figs. 34, 35) and appear as delicate parallel fibrils between the two opposite poles (figs. 37-39). These fibrils do not converge at the poles, and no centriole-like granules, as reported by some observers in other species, are seen at any stage. The achromatinic endosome of the nucleus remains often recognizable until the equatorial-plate stage (figs. 23, 37), when it disappears; it reappears after the completion of the division process (fig. 45). In the metaphase, four pairs of chromosomes apparently make up the equatorial plate (fig. 39). The precise mode of division of the individual chromosomes is unknown. In many cases the chromosomes appear to be of different sizes.

During the anaphase, two groups of chromosomes move apart and toward the opposite poles (fig. 40). Not infrequently there are seen very fine chromatinic threads connecting some of the separating pairs of chromosomes. In later stages, the chromosomes become collected near each of the poles (figs. 41-44), and finally two resting nuclei are reconstructed (fig. 45). In the meantime, the differentiated mass of cytoplasm surrounding the dividing nucleus elongates and finally divides into two masses, each containing one daughter nucleus. The spindle fibrils may remain stretched between the two separating nuclei for some time. Judging by the general appearance in smears, as well as in sections, these generative nuclei in specialized cytoplasmic masses are able to move about within the cytosome of the growing trophozoites (figs. 26, 28), appearing in various forms which suggest amoeboid movement.

Certain of the generative nuclei undergo a division which is quite different from that just described. The chromatin material becomes transformed into a spireme (fig. 46) which, however, presently differentiates into two chromatin threads (fig. 47). Toward the extremities of each of these two threads, the chromatin substance becomes condensed (fig. 48). As the nucleus elongates, the two dumbbell-shaped chromosomes become situated in the direction of the elongated nucleus (fig. 49), and each of the two pairs of chromosomes move apart; the connecting chromatin threads become stretched and finally break off (figs. 50, 51). At the end of the anaphase, the two chromosomes at each pole, which are often of

unequal size, do not undergo synchronous granulation, and consequently there appear more than two chromosomes at one or both poles (fig. 52). When finally the two nuclei are completely reconstructed (figs. 53, 54), the cytoplasmic division follows. The achromatinic endosome, which disappeared earlier (fig. 47), now reappears in one of the daughter nuclei (fig. 54). A similar division figure has often been seen in many species by various observers. Georgévitch (1936) saw stages similar to those indicated in figs. 48-51 in *Myxidium gadi* and interpreted them as phases in mitosis in which four chromosomes unite in two large reniform chromosomes before reaching the poles.

On the basis of the constant presence of one-half the number of chromosomes involved, as compared with normal mitosis, I consider this nuclear division as meiosis, by which the generative nuclei enter their last stage of activity prior to the initiation of spore-formation. Whether there is a second division cannot be stated, but I failed to see any figures suggesting the occurrence of that division.

Various stages in the development of spores have always been traced back to the forms shown in figs. 55-57. Imbedded directly in the cytoplasm (fig. 55), or often in a clear vacuole in the cytoplasm (figs. 56, 57), are two cells in association. Seen in sections, as well as smears, they show irregular outlines, though on the whole spindle-form, and bear a certain relation to the surrounding cytoplasm, as though possessing the capacity of amoeboid movement. Each cell contains a single nucleus, but the two nuclei in association are dissimilar in size and structure. The larger nucleus located in the larger cell shows in almost all cases a large achromatinic endosome (figs. 56, 58), while the smaller nucleus in the smaller cell usually does not contain such an endosome. This association may take place between the two newly divided cells, as shown in fig. 54, instead of between two cells produced by reduction division of different generative nuclei. That the latter is more common, is demonstrated by frequent occurrence of the two associated cells, as shown in figs. 55-57, in which the cells are coming in contact on lateral surfaces.

The two nuclei appear to remain independent and not to fuse with each other, at least during the early part of the period of spore-formation. The associated cells often leave a narrow space between them at the beginning (figs. 56, 58), but they soon become intimately united with each other (figs. 59-62), although the cell boundaries remain distinctly visible for some time. The nuclei divide presently. The division of the smaller nucleus may precede (figs. 57, 59, 62) or follow that of the large one (figs. 64-68), or the division of the two nuclei may be synchronous (figs. 60, 61). The nucleus preparing to divide shows an increase of chromatin substance which becomes transformed into a spireme (figs. 58-

60) that develops into two chromosomes. These chromosomes are most clearly noticeable during anaphase (figs. 59, 62, 64-66). In this mitotic figure, spindle fibrils are difficult to observe. The chromosomes become elongate (fig. 68), but condensed, as they approach the poles, and finally they are broken up into granules in the two daughter nuclei (figs. 63, 69). Thus sporonts with two large and two small nuclei are formed (figs. 69, 70). No achromatinic endosome is visible any more in any of the nuclei. As far as could be determined, the two smaller nuclei do not undergo further division but remain as the sporont (or pansporoblast) nuclei (figs. 78, 80), which may completely disappear when the spore-formation nears completion (fig. 81).

The other two nuclei become much enlarged and filled with chromatin material which is intensely positive to Feulgen's reaction (figs. 70, 71). During division the chromosomes are distinctly visible (figs. 72, 73, 75, 76). As the number of nuclei increases, the chromatin becomes more abundant and appears as heavy threads and coarse granules spread within the nuclei (figs. 74-76). In Feulgen preparations it is noticed that the nuclei are filled with chromatinic reticulum in which chromatin substance appears to be present in diffused state (figs. 77, 78). As the nuclear division continues, there appear in the sporont several compact chromatinic spherules which are occasionally vacuolated in the center, and at the same time the nuclei show a circular area which reacts negatively to Feulgen's reaction. These spherules, which also stain intensely with Heidenhain or Giemsa, have been noted in the developing sporonts of numerous species of Myxosporidia. Erdmann (1917) saw them in *Chloromyxum leydigi* and considered them to be glycogenous bodies; others called them "reduction nuclei." I have observed what I considered extrusion of plasmosomic endosomes at the corresponding developmental stages in the sporont of *Myxosoma catostomi* (Kudo, 1923, 1926). In the present myxosporidian, through the use of Feulgen's nucleal reaction, it has been established beyond doubt that Feulgen-positive substance is abundantly produced during the latter half of the period of development of the sporont and given off to the cytosome in the form of spherules about 0.5-1 $\mu$ in diameter. These chromatinic spherules are most frequently found in pairs (figs. 77-79), and in some, two spherules appear to have a connection between them, which suggests that these spherules may break up into smaller bodies by a simple fission. By the time the sporont differentiates into two sporoblasts (figs. 80, 81), these spherules disappear completely in almost all cases. Therefore, I am inclined to think as before (Kudo, 1926) that these chromatinic spherules are used for the formation of the spore-membrane and also of the polar capsules and their polar filaments.

In well-advanced sporonts containing ten or more nuclei, the nuclei become less chromatinic (fig. 79), and when fourteen nuclei are formed

they become definitely grouped in the two developing sporoblasts. Each sporoblast contains two nuclei for the sporoplasm, two for the polar capsules, and two for the valves of the spore membrane. Between the two sporoblasts there are usually visible two degenerating sporont nuclei previously referred to (fig. 80). Of the fourteen nuclei, ten degenerate gradually as the spore-formation proceeds. The four valve nuclei become ovoid, and characterized by a delicate chromatinic ring, while the four capsulogenous nuclei show various sorts of change in form and structure (fig. 80). However, the four sporoplasm nuclei remain normal in appearance and structure: namely, with a thick peripheral chromatin zone with granules and strands (figs. 80, 81). Although incompletely observed, the development of the polar capsules and filaments appears similar to that in *Leptotheca ohlmacheri* (Kudo, 1922). The developing polar capsules often react positively to Feulgen's reaction (figs. 81, 84, 85), showing the chromatinic origin of them, but when completely formed, they do not respond positively (figs. 89, 90). As to the shell-valves, no positive reaction to Feulgen has been noted, in contrast to species such as *Myxidium serotinum* and *M. immersum* (Kudo and Sprague, 1940).

The two sporoblasts continue to develop simultaneously and become transformed into spores. In young spores, the valve and capsulogenous nuclei remain visible for some time (figs. 82-86, 91), the latter persisting much longer than the former (figs. 89, 90). There are two nuclei in the sporoplasm of almost all spores (fig. 89), but in some the autogamous fusion appears to have already taken place (fig. 90). No phases of this union have been observed, but it is probable that a diploid nucleus is formed through karyogamy of two haploid nuclei. The sporont is in almost all cases disporoblastic as described here, but in a small number of cases it appeared to be monosporoblastic. The majority of trophozoites are disporous, and less frequently polysporous. Monosporous trophozoites are relatively rare.

Little was known about the nuclear phenomena at the time Gurley (1893) coined the term "pansporoblast" for "the transparent plasmasphere formed by the condensation of a portion of the plasma around one of the numerous nuclei of the endoplasm of the myxosporidium; in distinction from the sporoblasts which result from the segmentation of the pansporoblast." Mercier (1906) was the first to propose the occurrence of "phénomènes de sexualité" in the life cycle of *Myxobolus pfeifferi*. Since that time, numerous investigators reported autogamous nuclear fusion at some stages of development in various species of Myxosporidia.

Almost all investigators agree that the two sporoplasm nuclei undergo autogamy prior to, or after, the emergence of the sporoplasm as an amoebula. My observations on species of Myxosporidia, including the present form, also agree with this view.

The fact that mitosis occurs in Myxosporidia, became known as early as 1895 when Thélohan published his excellent treatise of these organisms. Mitosis of the trophozoite nuclei is now known in the following species: *Chloromyxum leydigi* (Naville, 1927); *Sphaerospora polymorpha* (Kudo, present study); *Sinuolinea dimorpha* (Davis, 1916); *Myxidium lieberkühni* (Bremer, 1922a); *Myxidium gadi* (Georgévitch, 1919, 1936); *Zschokkella rovignensis* (Nemeczek, 1922; Georgévitch, 1936); *Sphaeromyxa balbianii* (Naville, 1930); *Sphaeromyxa sabrazesi* (Debaisieux, 1924; Bělař, 1926; Naville, 1930; Georgévitch, 1936); *Myxosoma catostomi* (Kudo, 1926); *Thelohanellus swellengrebeli* (Schuurmans Stekhoven, 1919); *Myxobolus pfeifferi* (Mercier, 1908; Keysselitz, 1908; Georgévitch, 1936a); *Myxobolus destruens* (Schuurmans Stekhoven, 1920); *Myxobolus guyénoti* (Naville, 1928), *Henneguya gigantea* (Georgévitch, 1914, 1936). Each of these species has four chromosomes in its generative nuclei, except *Sinuolinea dimorpha,* which has six, according to Davis (1916). Six chromosomes were also claimed for *Sphaeromyxa sabrazesi* by Debaisieux (1924) and Bělař (1926), but later works (Naville, 1930; Georgévitch, 1936) show only four.

Concerning the starting point in the spore-formation, there are many views, even in one and the same species. Thélohan (1892, 1895) considered that each set of the two spores of *Myxobolus ellipsoides* and *M. pfeifferi* developed from a uninucleate generative cell which was "une petite sphère de protoplasma à contour net, qui semble limitée par une mince enveloppe résultant de la condensation de sa couche périphérique," and named it "la sphère primitive." Gurley renamed this body, as already stated, "pansporoblast." In this view, the sexual process is carried on in the sporoplasm, where the two nuclei fuse with each other. In recent years, such development was observed in *Sinuolinea dimorpha* (Davis, 1916), *Ceratomyxa coris* (Stempell, 1919), *Leptotheca ohlmacheri* (Kudo, 1922), and *Myxosoma ovalis* (Davis, 1923).

However, by far in a larger number of species of Myxosporidia, the initial stage of spore-formation is brought forward by association of two uni- or bi-nucleated cytoplasmic masses. Naville (1931) gave an excellent discussion of many views presented by various observers on this question. Therefore, I shall omit a general review. The two associating uninucleate generative cells in *Myxobolus pfeifferi* have been called macro- and microgametes by Mercier (1909), who believed that there was a complete fusion of both nuclei and cytoplasms. In *Sphaerospora polymorpha,* two uninucleate cells fuse without nuclear fusion, and in this respect the process resembles that found in *Myxobolus toyamai* (Kudo, 1917). The nuclear phenomena in the developing trophozoites of *Sphaerospora polymorpha* are somewhat similar, as a whole, to those of *Sphaeromyxa*

*sabrazesi, S. balbianii,* and *Myxidium incurvatum* as reported by Naville (1930), in that there are two types of divisions of the generative nuclei: namely, mitotic, to increase the number of the nuclei, and meiotic, to prepare for the formation of sporonts. However, I have not seen in *Sphaerospora polymorpha* the "4-microgamete stage" reported by Naville in Sphaeromyxa, which appears to have been seen also by Schröder (1907). Nor have I seen in the present species the so-called quartet in which two male and two female gamete nuclei become differentiated as reported by Naville in *Myxidium incurvatum.* In *Myxidium bergense,* Auerbach (1912) observed an association of two similar uninucleate cells to produce a binucleate sporont, somewhat similar to the one described here, but one of the nuclei is said to undergo reduction division before further development of the sporont takes place.

The view of Georgévitch (1923, 1936, 1936a), that the nuclei of the developing sporoblasts of *Myxidium gadi, Zschokkella rovignensis, Sphaeromyxa sabrazesi, Myxobolus pfeifferi,* and *Henneguya gigantea,* are all diploid, and that the two sporoplasm nuclei undergo meiotic division to become haploid prior to autogamous fusion, has not been confirmed by other investigators. Occasionally myxosporidian spores contain four nuclei, but because of their rarity one is led to think that this is an abnormal condition. Such was the case in *Myxosoma catostomi* (Kudo, 1926). In *Sphaeromyxa sabrazesi,* Debaisieux (1924) saw amitosis of the two sporoplasm nuclei, but Naville (1930) did not see a single instance of nuclear division in the sporoplasm. In *Sphaerospora polymorpha,* no spores contained either dividing nuclei or more than two nuclei in the sporoplasm.

## The Spore

The spore is spheroidal in general form (figs. 93-104). In front view, it is either circular or subcircular, with a slightly flattened anterior margin (figs. 94-96, 100). In end view, it is circular or broadly ovoid and somewhat pointed along the sutural line (figs. 97, 98). In side view, it is oval with a slightly pointed sutural ridge (fig. 99).

The spore membrane is uniformly thick, and composed of two valves which are sometimes asymmetrical. The sutural ridge is distinctly visible in life (figs. 88, 92, 96-104), and coincides with the broadest plane of the spore, so that it cannot be seen in front view (figs. 94, 95). Each of the two polar capsules is, as a rule, connected with the anterolateral margin of the valve close to the sutural plane (figs. 94-96). In many spores the sutural ridge takes unusual or abnormal courses. Most frequently the ridge makes some acute angles with the broadest plane of the spore, so that it can be seen in front view (fig. 87). In a number of spores the sutural ridge is at right angles to the largest axis of the spore, one polar

capsule being located on each side of it (fig. 100). Such spores resemble those of Leptotheca. The surface of the membrane shows, without exception, numerous fine ridges or elevated striae, which give the spore a striated appearance. All striae appear to be uniformly high and thick, unlike those found in certain other myxosporidians, such as *Leptotheca ohlmacheri* (Kudo, 1922), *Myxidium serotinum* (Kudo and Sprague, 1940). There are about 20 to 30 striae on each valve, which are, as a rule, parallel to one another and to the sutural ridge (figs. 87, 92, 96-98, 100, 101). In many spores, however, these striae are not parallel to the sutural line and make various angles with it (figs. 88, 93, 102-104). In still others, there are 2 or 3 ridges which encircle the margin of the valve, and numerous parallel ridges are evenly distributed over the entire valve-surface (fig. 99). Davis (1917) in his description states: "On each side are a number of concentric striations extending around each valve parallel to the sutural line."

There are two polar capsules situated in the anterior half of the spore. They are of nearly the same size, although in a few spores unequal capsules are found. They are pyriform in shape and contain a polar filament which is coiled 3 to 6 times and which is clearly visible in life. The neck of the capsule is drawn out into a fine tube and opens in the shell-valve at the anterolateral margin near the sutural ridge (figs. 91, 92, 94-96). Thus the two polar capsules are extremely divergent in front view. There is one foramen for the capsule on each valve, a condition seen in many other species, as for example, in *Leptotheca ohlmacheri* (Kudo, 1922), *Myxidium immersum* and *M. serotinum* (Kudo and Sprague, 1940).

This divergency of the two capsules is quite uncommon except in the family Myxidiidae. In his original description of the species, Davis (1917) stated: "capsules large, distinctly pyriform. Coiled filaments distinct." In his figs. 88 and 91, Davis showed spores, the two polar capsules of which are nearly parallel to each other, while in those shown in his fig. 89, the two capsules are slightly convergent or parallel to each other. In no one of the spores did Davis figure the divergent capsules. This consistent difference in the position of the polar capsules between Davis' species and my own material puzzled me for some time. Recently, through the kindness of Dr. Davis, I examined two smears of the contents of the bladder of the toadfish that had been prepared by him at the time of his investigation (1917). The smears were fixed with osmium vapor, and stained with Heidenhain's and Delafield's haematoxylin. The Heidenhain preparation showed still distinctly stained polar capsules in developing spores, while mature spores in the smear were too intensely stained to allow any observation. The young or newly matured spores showed in every case

divergent polar capsules, which condition is clearly visible in front view. Therefore, it is held that the normal spore of *Sphaerospora polymorpha* possesses two divergent polar capsules. In this respect, the present species resembles strikingly *Sphaerospora divergens* as observed by Thélohan (1895) and Auerbach (1912).

Occasionally spores with one, three, or four polar capsules (fig. 19) are encountered, which conditions appear to have been brought about by abnormal arrangement of the capsules during the development of disporoblastic sporonts.

The polar filaments are easily extruded from the capsules in fresh spores, when the latter are subjected to mechanical pressure or treated with potassium hydrate or hydrogen peroxide, as I have experienced in my previous studies with various Myxosporidia. When fully extruded, the polar filaments measure 24 to 32 $\mu$ in length, and in some cases the tubular nature of the filament could be noticed (fig. 93).

The main mass of the sporoplasm is situated in the posterior half of the spore, but extends anteriorly between the capsules and expands along the anterior margin of the spore (figs. 94-96). The sporoplasm consists of finely granulated cytoplasm, and contains two vesicular nuclei which are usually noticeable in life (fig. 94).

When the spores are fixed and stained, the characteristic feature of a bicapsulated myxosporidian spore becomes plainly visible (figs. 89-91). The valve nuclei may be seen in young spores as delicate rings in Feulgen's preparations (figs. 83-85) or as thickenings in the membrane in Heidenhain or Giemsa preparations. However, they disintegrate much earlier than the capsulogenous nuclei which remain in close association with the developing capsules (figs. 89-91). In some cases the capsulogenous nuclei may remain recognizable even after the two sporoplasm nuclei fuse (fig. 90). The sporoplasm nuclei are almost always two in number and rich in chromatin substance (figs. 84, 85, 89, 91).

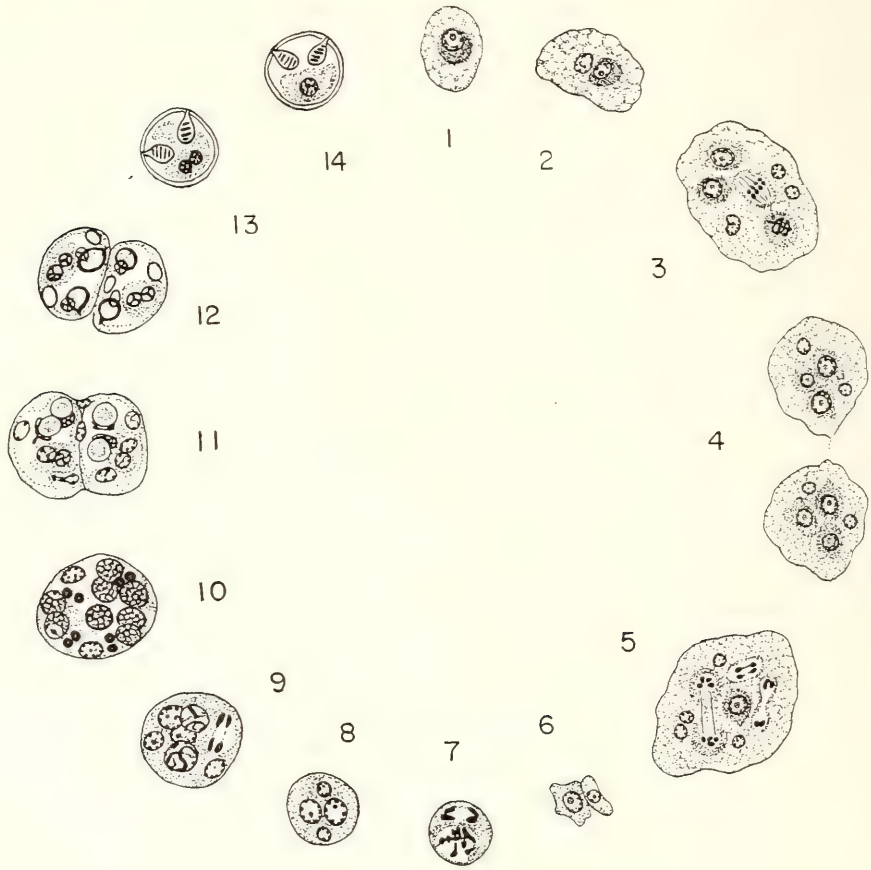
The fine striae or ridges present on the outer surface of the spore membrane appear in Giemsa smears to be composed of small granules arranged in linear series at the beginning (figs. 87, 88), but to be continuous fine lines when fully formed (fig. 92).

The fresh spores as viewed in the host's urine or salt solution vary in dimensions. A few small spores measure only 6.5 $\mu$ long and wide, and about 5-6 $\mu$ thick. The largest spores measure 11 $\mu$ in diameter. The majority of the spores however are 7.5-9.5 $\mu$ in length and breadth and 7-8 $\mu$ in thickness. The polar capsules are 3.8-5 $\mu$ long by 2-2.8 $\mu$ in diameter. Davis (1917) gave the following dimensions of the spores of *Sphaerospora polymorpha,* which he measured presumably in fresh condition: "diameter of spore 7-10 $\mu$, averaging about 8 $\mu$; capsules 2-2.5 $\mu$ by

4-5 $\mu$." As in the case with other species (Kudo, 1921c), fixed and stained spores in permanent smears show smaller dimensions. Spores fixed with Carnoy, Schaudinn, or Bouin, stained with Heidenhain or Giemsa or subjected to Feulgen's reaction, and mounted in Canada balsam or cedar wood oil, average 6.2-8.5 $\mu$ long and wide and 5.5-7.5 $\mu$ thick, and the polar capsules were 3-4 $\mu$ long by 1.5-2.5 $\mu$ in diameter.

### Résumé of Development of *Sphaerospora polymorpha*

The development of the trophozoite and the process of spore-formation in *Sphaerospora polymorpha,* as observed in the urinary bladder of *Opsanus tau* and *O. beta,* will be summarized briefly here (text fig. 6). The youngest stage is a uninucleate trophozoite in which the nucleus is surrounded by a differentiated cytoplasmic mass (1), which gives rise




Text fig. 6.—Diagram showing the development of *Sphaerospora polymorpha* in the urinary bladder of the host fish.

to a binucleate trophozoite that contains a generative and a vegetative nucleus (2). The generative nucleus multiplies by mitosis, during which change four chromosomes appear; the vegetative nucleus divides by amitosis (3). Simple plasmotomy increases the number of trophozoites (4); multiple plasmotomy probably occurs also. The generative nuclei undergo reduction division (5) to produce uninucleate generative cells, each with a haploid nucleus, which become associated in pairs to produce a sporont or pansporoblast (6). The cytoplasmic masses fuse, but the two nuclei remain independent, and both undergo division, in which two chromosomes appear (7, 8). The division is repeated (9). The chromatinic substance of the nuclei increases, and small spherules composed of the same substance appear in the cytosome of the developing sporont (10). Finally the sporont becomes differentiated into two sporoblasts, each containing six nuclei (11), and each sporoblast develops into a spore (12). Mature spores first contain two haploid nuclei (13), and then a single diploid nucleus. The gap between 14 and 1 is unknown at present.

## Taxonomic Consideration

At the time when Davis (1917) described *Sphaerospora polymorpha,* there were six known species of the genus (Kudo, 1920a). Of these, *Sphaerospora divergens,* which Thélohan (1895) found at Concarneau and Roscoff, France, in the renal tubules of *Blennis pholis* and *Crenilabrus melops,* appears to possess the spore which resembles that of the species under consideration. The spores of *S. divergens* are spherical and measure 10 $\mu$ in diameter, sometimes 10 $\mu$ by 12 $\mu$. The spore membrane is marked by striae difficult to recognize. The polar capsules are divergent; polar filaments are visible in life, and when extruded under the action of potassium hydrate measure 20-25 $\mu$ in length. This myxosporidian was further observed by Parisi (1912) in the kidney of *Crenilabrus pavo,* at Naples, Italy. Auerbach (1912) found it in the urinary bladder of *Hippoglossoides limandoides* in Norway, and showed that the spores were 10 $\mu$ in diameter by 8 $\mu$ thick. About 4 $\mu$ long polar capsules are divergent, and the spore membrane shows a fine striation. In his figures, Auerbach indicated that in front view there are about 20 fine parallel striae in two groups on the shell, and in side view seven and nine parallel striae meet the sutural ridge at acute angles. Jameson (1931) reported this species in the urinary bladder of *Pleuronichthys verticalis* of San Pedro, California, and stated that the spores were slightly smaller than those previously reported from European waters, being 8-10 $\mu$ in diameter.

Thus it appears that the spores of these two closely resembling species differ in dimensions. Moreover, the host species are distinctly different, although we do not possess much data as to how much this host-specificity

could be depended upon for taxonomic purposes. I think Davis (1917) was justified in naming *Sphaerospora polymorpha* as a new species.

It may be noted here that since 1917, the following eight species of Sphaerospora have been described: *S. gasterostei* (Schuurmans Stekhoven, 1920), *S. tincae* (Plehn, 1925) (= *S. pernicialis,* Léger, 1929), *S.* sp. (Davis, 1917), *S.* sp. (Southwell and Prashad, 1918), *S.* sp. (Kudo, 1920a), *S. subelegans* (Fantham, 1930), *S. renalis* (Bond, 1938), and *S. notropis* (Fantham, Porter, and Richardson, 1939). None of these species possesses the spore similar to that of the present species.

### Diagnosis of *Sphaerospora polymorpha* Davis

*Habitat.*—In the urinary bladder of *Opsanus tau* and *O. beta.* Beaufort, North Carolina (Davis); Solomons Island, Maryland; Englewood, Florida (Kudo).

*Trophozoite.*—Amoeboid, with conical pseudopods from limited body surface; usually attached to bladder epithelium, rounded; larger forms elongate. Cytosome differentiated into ecto- and endo-plasms; with numerous fat globules. Simple and multiple plasmotomy. Size up to 100 $\mu$ long by 20-65 $\mu$ wide; the majority range from 20 to 50 $\mu$ in length. Sporont disporoblastic or rarely monosporoblastic. Trophozoite disporous, polysporous, or, rarely, monosporous.

*Spore.*—Spheroidal; sutural ridge distinct; shell-valves symmetrical, finely striated; striation in many cases parallel, but may be at various angles to sutural line. Polar capsules conspicuously divergent. Fresh spores: 7.5-9.5 $\mu$ in diameter, 7-8 $\mu$ thick. Polar capsules: 3.8-5 $\mu$ long by 2-2.8 $\mu$ wide. Fixed, stained, and mounted spores: 6.2-8.5 $\mu$ in diameter by 5.5-7.7 $\mu$ thick; polar capsules 3-4 $\mu$ by 1.5-2.5 $\mu$.

*Remarks.*—It is extremely common, but does not bring about any noticeable pathological effect on the host fish. The spore of this species resembles closely that of *Sphaerospora divergens.*

### Mode of Infection

Although, as was pointed out before, there are numerous species of coelozoic Myxosporidia which inhabit the urinary bladder, ureters, or renal tubules of the kidney of host fish, no experimental evidence is available to indicate how the infection begins. In the case of *Leptotheca ohlmacheri,* which is parasitic in the lumen of the uriniferous tubules of the kidney of frogs and toads, Ohlmacher (1893) wrote: "as to the origin of the myxosporidian infection, we can only conjecture that it must have occurred by way of the cloaca to the bladder, eventually lodging in the kidneys." I have already pointed out that this view is untenable "because

myxosporidian spores have no power of locomotion and have never been seen or been made to germinate in water outside of the host" (Kudo, 1922). Experimental germination of the spores in the pylorus or duodenum of the frog suggested that the liberated amoebulae probably penetrate through the wall of the digestive tract, appear in the coelomic fluid, and finally reach the uriniferous tubules.

Regarding *Sinuolinea dimorpha,* parasitic in the urinary bladder and Wolffian ducts of *Cynoscion regalis,* Davis (1916) observed that "spores when placed on the slide without previous exposure to sea water, and mixed with a drop of fluid from the pyloric caeca of the host, usually germinated within five to fifteen minutes," and suggested that "it appears probable, therefore, that the free spores, when taken into the intestine of the host, germinate, and the sporozoites, as free amoebulae, actively make their way into the urinary bladder."

In the toadfish, the digestive canal and urinary organ are not directly connected at any point, and there is no possibility of the amoebulae which become free in the intestine leaving the anal opening and entering the urinary bladder. It is therefore suggested that the amoebulae emerge from the spores in the intestine, penetrate through the gut wall, and enter the coelom, finally reaching the urinary bladder. Exact information as to how this myxosporidian reaches the bladder remains to be obtained through experimental infection in the future.

## V. *NOSEMA NOTABILIS* KUDO

### Occurrence

In order to ascertain whether the host fish tissues were infected by the microsporidian, serial sections of the kidneys, ureters, and gonads of eleven fish were carefully examined. The urinary bladders of these fishes not only harbored large numbers of the trophozoites of *Sphaerospora polymorpha* in which *Nosema notabilis* was abundantly present, but also contained many spores of this microsporidian floating freely in the urine. Up to the present time, however, this microsporidian has not been observed in any of the host tissue cells. In all fishes examined, many helminths were found in various viscera. Since several helminths are known to be hosts for microsporidians (Kudo, 1924b), it was natural to suppose that there might be some individuals of these worms which were infected by *Nosema notabilis.* Although many worms taken from viscera in close association with the urinary system of the host fish were examined in smears and sections, none showed any infection. Therefore, I am led to hold that *Nosema notabilis* is a specific and exclusive parasite of the myxosporidian *Sphaerospora polymorpha.*

The Sphaerospora trophozoites present in all host fishes examined, were infected in turn by *Nosema notabilis* to a varying extent. When the infection was intense, isolated spores could easily be detected in the urine of the fish bladder in an ordinary fresh smear (fig. 160). In cases of slight infection, there were few free spores, and it was necessary to search several microscopic fields before finding one. In one of the heaviest infections, it was estimated roughly that more than 75 per cent of the myxosporidian trophozoites present in the bladder were infected by *Nosema notabilis* (figs. 163, 164, 175), and in the lightest infection, less than one per cent of the myxosporidian trophozoites appeared to be infected. Because of the strong refractility, the mature spores of the microsporidian are easily recognized in life under a comparatively low magnification. On the other hand, the detection of schizogonic as well as sporogonic stages in life can only be made out with an oil immersion objective and proper illumination.

Host trophozoites of various dimensions are all infected by the microsporidian. However, trophozoites which are 50 $\mu$ or more in length are seldom seen heavily infected, and those which are less than 40 $\mu$ in diameter are most frequently heavily loaded with the microsporidians (figs. 8, 147-158, 163, 164, 173-175). In the heavily infected host trophozoites, the host nuclei appear to have degenerated (figs. 154, 158, 173) or hypertrophied (figs. 150, 151), and there is a complete inhibition of sporulation processes. These changes would most probably result in the total degeneration and disintegration (fig. 174) of the host bodies, which then would set free the microsporidian spores in the urine. Perhaps this accounts for the absence of heavily infected large trophozoites, as the infection brings about degeneration before the hosts are able to grow into larger trophozoites. Undoubtedly, the microsporidian can be considered as pathogenic to the host myxosporidian.

It is occasionally difficult to determine in life whether the Nosema spores present in a small host trophozoite developed in, or were ingested by, the latter, especially when only a few spores are involved. The four spores of *Nosema notabilis* shown in fig. 9 appeared fully normal in appearance, and therefore are considered to have developed within this host trophozoite. More or less heavily infected host trophozoites are usually without pseudopodia and are invariably rounded (figs. 8, 9), although uninfected individuals present in the same field show distinct pseudopodia (figs. 1-7).

Léger and Duboscq (1909) discovered *Nosema frenzelinae*—the very first authentic record of microsporidian infection in a protozoan—in *Frenzelina conformis,* a cephaline gregarine, parasitic in the intestine of *Pachygrapsus marmoratus* at Cavaliere, France. This microsporidian was found in the cysts as well as sporadins of all ages of this gregarine.

Ordinarily, almost all the gregarines present in a crab were infected, although not all crabs harbored infected host protozoans, and the host crab tissues were free from infection. The infected gregarines grew and encysted in pairs; the nuclei underwent division, but gametogony did not take place, which the two authors referred to as a phenomenon of parasitic castration in a protozoan.

The same authors (1909a) further noticed another microsporidian, *Perezia lankesteriae,* in an acephaline gregarine, *Lankesteria ascidiae,* occurring in the intestine of *Ciona intestinalis* at Cette, France. In this case also, the microsporidian was exclusively gregarinophilous and did not invade the Ciona tissues. Moreover, only adult cephalins or sporadins which were extracellular were infected, all intracellular forms being free from the microsporidian.

In the case of *Nosema marionis* (Thélohan) which, according to Stempell (1919), is an exclusive parasite of the myxosporidian *Ceratomyxa coris* Georgévitch (1916), inhabiting the gall bladder of *Coris julis* and *C. giofredi* in coastal waters of France, Georgévitch (1917), who held that the two cnidosporidians were co-existing in the amoeboid individuals "par la plasmogamie accidentelle des plasmodies de ces deux parasites," remarked that in a large proportion of the two parasites both organisms underwent sporulation, which suggests that the microsporidian infection in the Ceratomyxa trophozoites was quite common and harmless. Stempell (1919) who found that the association of the two cnidosporidians was in reality one of parasitism, states that *Nosema marionis* is a common parasite of *Ceratomyxa coris.* Stempell studied the two host species of Coris and found that of 36 Ceratomyxa-infected fish, 15 contained trophozoites of *Ceratomyxa coris* that were infected at least in part by *Nosema marionis.* As to the effect of infection on the host Ceratomyxa, Stempell observed that since the spore-formation of *Ceratomyxa coris* was not affected by *Nosema marionis,* even when the infection was heavy, the microsporidian did not seem to harm the host myxosporidian to any noticeable extent. Thus it appears that *Nosema notabilis* is the sole true pathogenic microsporidian parasite in protozoa.

## Schizogony and Sporogony

The earliest development stage of *Nosema notabilis* has been found in small host trophozoites. It was a minute binucleate body which measured 0.5-2 $\mu$ in diameter. The irregular outline of its body indicates that it probably is able to undergo amoeboid movement (fig. 116). It resembles very closely the sporoplasm of the spores which have extruded the polar filaments under experimental conditions (figs. 114, 115). This binucleate schizont divides by a binary fission into two uninucleate schizonts (figs. 116-120, 155-157). Obviously division is repeated (figs. 121-127, 150).

When the host trophozoites multiply by plasmotomy, the daughter trophozoites remain infected by the microsporidian.

The nuclei, which respond positively to Feulgen's nucleal reaction, are compact granules without any recognizable internal differentiation. The nuclear division is seemingly amitotic, as observed in various species of Nosema in the past. When the uninucleate schizonts enter the second phase of development, the body becomes enlarged, and assumes a spindle-form with first short (figs. 128, 129) and later much drawn-out ends (figs. 130-133). As the body grows, the conspicuous, compact nucleus also enlarges itself and undergoes an amitotic division which proceeds slowly, so that various phases are quite frequently observed (figs. 129-131, 151, 152). The individual nuclei are compact and often appear more or less triangular in shape, with the bases facing each other. The clear zone which was present around the parent nucleus becomes enlarged and elongates as the nuclear division progresses, which indicates an increase in the amount of nucleoplasm at this stage. In highly flattened smears, the nucleus of the schizont is often greatly spread out (fig. 134) and shows 7 to 10 minute chromatin granules with connecting threads, but its division is clearly amitotic (figs. 135, 136, 152).

In *Nosema marionis,* Stempell saw a direct nuclear division which he stated could be called a primitive karyokinesis, since he noticed the ends of the dividing nucleus were characteristically pointed, and in what he designated as an anaphase there was a "wurstförmige Verdickung" between the division plane and each of the poles. Similar appearances were noticed in *Nosema notabilis* (fig. 151), but I find no ground to maintain that the division is indirect. The spindle-form schizonts have previously been seen in numerous species of Microsporidia, such as *Nosema bombycis* (Stempell, 1909; Kudo, 1916; Foà, 1924), *Stempellia magna* (Kudo, 1925a), *Nosema marionis* (Stempell, 1919), *Thelohania legeri* (Kudo, 1924), *Nosema nonagriae* (Schwarz, 1929), and *Thelohania ephestiae* (Mattes, 1928). In *Nosema binucleatum,* Weissenberg (1926) observed these forms in abundance. He considered the individuals ("Schlauchzellen") such as shown here in figs. 134-136 and 153 degenerating forms. I am inclined to think that they are extremely spread-out normal schizonts rather than degenerating individuals.

The two nuclei of spindle cells move towards the opposite extremities which continue to grow out (figs. 130, 131), and the central portion becomes constricted (figs. 132, 133). In the meantime, each nucleus undergoes a division once more (figs. 137, 138). The two schizonts may now separate or remain together (figs. 139, 141), and in a comparatively small number of cases, each of these daughter schizonts may divide again, or the two nuclei in one daughter schizont may move apart and undergo similar changes, resulting in the formation of three binucleate pyriform

schizonts, which remain in a chain (fig. 140). This chain formation has been reported previously in several species of Microsporidia.

Finally, binucleate sporonts (figs. 143, 144) are formed, which transform themselves directly into sporoblasts, and in turn into the spores. The number of nuclei remains two throughout these changes. The cytoplasm draws away first from one end (fig. 145) and then from the other, thus forming the girdle-shaped sporoplasm (fig. 146). How the remarkably long polar filament becomes developed in a comparatively short time is entirely unknown in the present species.

In all Microsporidia in which the process of spore formation has been observed, the sporoblast is either uni- or bi-nucleate. A number of workers, as Mercier, Stempell, Léger and Hesse, etc. (Kudo, 1924a), had been inclined to think that during the development of a sporoblast into a spore, there occur further divisions or breaking up of the nuclei, some remaining in the sporoplasm, others controlling the formation of the spore membrane and polar filament. For example, Stempell (1919) maintained that the development of the spore of *Nosema marionis* was similar to that of *N. bombycis* (Stempell, 1909), and concluded as follows: "Es scheint, als ob auch hier schliesslich sieben Kerne, d.h. vier Amoeboidkeimkerne, zwei Schalenkerne und ein Polkapselkern entstehen." His figures showing this so-called nuclear division, appear to be simple binucleate sporoblasts or young spores as seen in *N. notabilis*. Stempell's fig. 64 especially, which is supposed to illustrate the 7-nucleate sporoblast, is far from convincing. Fantham (1939) still holds the view which he expressed in his study of *N. apis* that the single nucleus in the sporoblast of *N. cactoblastis* and *N. cactorum* divides into five nuclei (two for the membrane, two for the sporoplasm, and one for the polar capsule and filament). Here, also, there is insufficient evidence to support the view. These changes are similar to the process of spore-formation clearly observable in all Myxosporidia, but there is no evidence to justify such a comparison. In *Thelohania legeri* and *T. indica* (Kudo, 1929), the nucleus of the sporoblast divides in two, and one part seems to be concerned with the formation of the polar filament, while the other remains as the sporoplasm nucleus. In *Stempellia magna* (Kudo, 1925a), an unusually large spore-producing microsporidian, and *Nosema aedis* (Kudo, 1930), the sporoplasm contains a single nucleus. In *Stempellia magna,* with the condensation of the sporoblast cytoplasm towards one end, and appearance of a large clear space towards the other, there appear granules which stain less intensely than chromatin material and which apparently become transformed into the polar filament (Kudo, 1925a). In his studies of various Microsporidia by means of Feulgen's nucleal reaction, Jírovec (1936a) did not observe any division of the nuclei of the sporoblast. Trappmann (1923, 1926) noticed in Giemsa-stained preparations of *Nosema apis* that the two nuclei of a sporoblast

segment off parts which wander into the posterior vacuole in granule form and join with fine cytoplasmic strands to produce the polar filament. With Feulgen's reaction, Jírovec showed that during this change in *N. apis,* the sporoblast as well as the spore were binucleate, and there was no visible nuclear participation in the formation of either spore membrane or polar filament.

During the spore-formation of *Sphaerospora polymorpha* (p. 25) and numerous other Myxosporidia, each polar capsule originates within a special uninucleate capsulogenous cell, and the filament is formed under the control of the nucleus (Kudo, 1922). Upon complete development of the capsule and its filament, the nucleus may still remain as such, though it finally degenerates completely. The polar filament of Myxosporidian spores is comparatively short, though much larger in diameter. The polar filament of *Nosema notabilis* and other microsporidians is relatively much longer and very much finer than that of a myxosporidian. One may suppose, therefore, that it develops during the maturing of the spore, under the control of a special nucleus. In fact, several observers have reported such a nucleus, but it has not been seen in cases where the developing sporoblasts were subjected to Feulgen's nucleal reaction.

In the sporoblasts of *Nosema notabilis,* I have examined numerous individuals which were subjected to Feulgen's nucleal reaction or were stained with Giemsa's solution or Heidenhain's haematoxylin, but I have failed to find any nuclear structure in addition to the two nuclei of the sporoplasm. Since the two nuclei are quite dissimilar in size and form, one may assume that one controls the formation of the membrane and the filament, while the other remains as the generative nucleus.

The early phases of the development of Microsporidia have not been seen in many species. In a few instances of experimental infection, certain portions of the development have been seen, but in no case has observation in life been carried through. In *Stempellia magna,* the spores are large, and I was able to observe part of the early developmental phase in life (Kudo, 1925a). The uninucleate sporoplasms emerge from the spores as amoebulae and apparently penetrate through the gut epithelium of the host Culex larva, although the actual penetration was not seen. In the case of *Nosema bombycis,* Stempell (1909) maintained that the emerged binucleate amoebulae become uninucleate bodies by fusion of the two nuclei. He coined the term "planonts" for these 0.5-1.5 $\mu$ large bodies which were said to multiply rapidly: "Sehr häufig liegen die jungen Planonten auch noch in der Nähe leerer Sporenhüllen, sind aber meist bereits in lebhafter Vermehrung durch Zweiteilung und Knospung begriffen, so dass man gewöhnlich ganze Nester antrifft." Stempell thus maintained that the planonts undergo divisions in the fore- and mid-gut lumen one or two days after ingestion of spores and also in the "bloodstream" of *Arctia caja.*

With respect to *N. marionis,* Stempell (1919) states that its development is similar "im wesentlichen nach dem Schema" for that of *N. bombycis.* In this case, Stempell observed all stages in the cytosome of host trophozoites and added no further information except that the earliest stage was a uninucleate planont. No students of Microsporidia have been able to confirm Stempell's view that the two nuclei in the amoebula fused into one before beginning development. However, a number of workers reported the same changes in other species of Microsporidia, which were entirely based upon stained smears and sections. For example, Trappmann (1926) by studying stained smears of *N. apis,* makes the following statement:

> Der aus der Spore ausgeschlüpfte Planont ist amöboid beweglich und zeigt deutlich zwei Kerne. Durch Verschmelzen der beiden Kerne (Autogamie) treten bald einkernige Planonten auf, die bei Neuinfektionen in Ausstrichpräparaten wiederholt gefunden wurden. Schon im Planontenstadium ist der Parasit in der Lage, sich durch successive Zweiteilung stark zu vermehren und grössere Kolonien hunger Planonten zu bilden, die Man auf Schnitten in den Falten des Mitteldarmes finden kann. Die Planonten wandern zu den Epithelzellen des Mitteldarmes hin und dringen durch den Stäbchensaum in diese Zellen ein.

Stempell's original idea seems to be based on an analogy with the early development of myxosporidian spores, in which autogamous union of the two nuclei of the sporoplasm takes place. Trappmann seems to have simply followed this idea without showing concrete evidences for so doing. The majority of recent workers engaged in solution of this phase of microsporidian development are uncertain about the nuclear change at this stage, since living organisms cannot continuously be observed. In connection with the early development of *Plistophora blochmanni,* Zwölfer (1926) makes the following statement:

> Es war anzunehmen, dass die Amöboidkeime diese leeren Hüllen bereits verlassen hatten und im Darmlumen frei auftraten. In der Tat konnte das Vorhandensein einer grösseren Anzahl von kleinen, rundlichen bis ovalen im ganzen etwas unregelmässigen Gebilden im Darm festgestellt werden. Sie lagen teils dicht neben leeren Sporenhüllen, teils in einem geringen Abstand von solchen (Taf. 13, Fig. 33). Nach Grösse und Aussehen stimmten diese Körper völlig mit jenen Amöboidkeimen überein, die kurz vor dem Verlassen der Sporenhülle standen. Ich deute sie daher als ausgeschlüpfte freie Amöboidkeime. Diese Gebilde waren ein- oder zwei-kernig. Im ganzen überwogen die zweikernigen Formen. Bei einigen zeigte die chromatische Substanz einen mehr aufgelockerten, bei anderen wiederum einen mehr kompakten an die Verhältnisse im ruhenden Sporenkeim erinnernden Bau. Irgendwelche Anhaltspunkte, dass eine Verschmelzung der beiden Kerne stattfindet, dass somit die freien einkernigen Amöboide aus den entsprechenden zweikernigen hervorgehen, habe ich nicht gewonnen. Auch habe ich keinerlei Beobachtungen gemacht, dass die zweikernigen freien Amöboide einer Teilung ihres Protoplasmakörpers durchmachen.

Schwarz (1929), who also made a careful study of *Nosema nonagriae,* states:

> Der Amöboidkeim der Spore behält anschneinend nach dem Ausschlüpfen seine beiden Kerne bei (Stadium 1). Bevor nun die vegetative Vermehrung durch Schizo-

gonie eintritt, glaube ich das einkernige Stadium (2) annehmen zu können. Jedoch fehlt mir dafür ein exakter Beweis. Die beiden Kerne des Amöboidkeimes würden nach dieser Annahme also zu einem verschmelzen.

In *Nosema binucleatum,* Weissenberg (1926) saw no nuclear fusion in the binucleated amoebula, and Mattes (1928) believed that the emerged binucleate amoebulae of *Thelohania ephestiae,* found among empty spores in the haemocoele or in newly infected fat bodies of the host, remained binucleate.

In my study of *Nosema bombycis* (Kudo, 1916), I did not find the planont stages described and figured by Stempell, in which a uninucleated round body increases rapidly in number by division. Foà (1924), also working on the same microsporidian, did not find living individuals in this stage, since she wrote:

> Non mi è stato possibile vedere in nessun caso nè in strisci di emolinfa, nè in sezioni di bachi, quella divisione dei planonti che nessuno, dopo Stempell, ha riscontrato. Mi sembra che le figure relative di Stempell (125-126-127) siano troppo poco dimostrative; si aggiunga che sono tolte dall'*Arctia caja* e non dal baco da seta. Ritengo che il planonte diventi subito intracellulare cioè si transformi in meronte, senza dividersi prima, e che appena fissato o poco prima di fissarsi diventi uninucleato per la fusione dei due nuclei.

However, no students of Microsporidia doubt the presence of extracellular stages between the emerged amoebulae and intracellular schizonts. Trager (1937) apparently carelessly misinterpreted the planonts as merely extracellular forms, instead of the original meaning of the term as coined by Stempell. The fact that his silkworm tissue culture contained "wandering cells full of parasites" indicates simply that there is an extracellular stage of the organism following the emergence from the spore, as has been believed by all, and there is no evidence at all to show that actively multiplying planonts of Stempell were present in the haemocoele. Therefore, the following statement made by Trager is clearly incorrect: "The one successful case proves unequivocally the presence, in the blood of silkworms which have recently ingested spores, of minute extracellular infective forms, the 'planonts' of Stempell, the proof for the existence of which has been doubted (Kudo) and has heretofore rested solely on morphological evidence."

## The Spore

The mature spores are ovoid to ellipsoid, with unequally rounded extremities (figs. 105, 160). When a mature spore is examined in life, it may be composed of a homogeneous protoplasm which fills the spore cavity (figs. 105, 160), or when focused through the axis, it may show a clear space at the more rounded end (figs. 106, 107). Though ordinarily called a vacuole, it invariably shows a minute granule, and in certain spores, a filamentous structure spreading from side to side is also notice-

able. Quite frequently, nearly mature spores appear pyriform, with a comparatively large clear space near the broader extremity (fig. 107). In such a spore, several transverse striae may be found in the remaining portion of the cavity.

The spores vary more or less in size. The average fresh spores measure 3.3 $\mu$ long by 2 $\mu$ in diameter; but the length varies from 2.9 to 4.8 $\mu$, and the width, from 1.4 to 2.5 $\mu$. The smallest spores measured 2.9 $\mu$ by 1.4 $\mu$, and the largest, 4.8 $\mu$ by 2.5 $\mu$. Extreme diversities in size and form of the spores of the same species of Microsporidia are not uncommon; for example, the spores of *Nosema marionis* were reported by Stempell (1919) as varying from 1.5 to 7 $\mu$ in length, while Thélohan recorded 8 $\mu$ as their length.

As in the great majority of Microsporidia, the internal structure cannot be made out in fresh spores, so that it is necessary to resort to fixation and staining. The spore membrane of *Nosema notabilis* is of uniform thickness and composed of a single piece, as found in the majority of microsporidians.

When the spores are fixed with Schaudinn's or Bouin's fluid and stained with Heidenhain's iron haematoxylin, a band-shaped ring of variable width and form becomes deeply stained in the middle of them (fig. 108). Slightly to one side of the anterior tip, there is invariably seen a compact granule from which extends towards the band a delicate less deeply stained filament. This granule, that is apparently the basal thickening of the polar filament, has been seen in the spores of numerous species. It has been noticed in *Nosema bombycis* (Stempell, 1909; Kudo, 1916), *Plistophora macrospora* (Léger and Hesse, 1916), *Plistophora simulii* (Debaisieux and Gastaldi, 1919; Debaisieux, 1928), *Glugea anomala* (Debaisieux, 1920), *Thelohania legeri* (Kudo, 1921a), *Stempellia magna* (Kudo, 1925a), *Plistophora blochmanni* (Zwölfer, 1926; "Polkörper"), *Thelohania ephestiae* (Mattes, 1928), *Nosema nonagriae* (Schwarz, 1929), *Perezia legeri* and *P. mesnili* (Paillot, 1929).

Posterior to the band is an area which is still less deeply stained and is delimited by a filamentous border (fig. 108). When the preparations are further decolorized, the less deeply stained portions noted above become completely destained and unrecognizable, and the band-ring is now seen stained lightly, revealing two compact short rod-shaped nuclei which are deeply stained (fig. 109). Such a spore if seen endwise shows clearly that the band-form protoplasm is a ring with two nuclei imbedded in the periphery (fig. 110).

When the spores are stained with Giemsa's solution, the band-ring of varying width, containing two compact nuclei, becomes conspicuously noticeable, while the parts between this band and the two extremities remain unstained as "vacuoles" of various shapes (figs. 113, 152, 158).

In some spores, the filamentous structure extending between the band and a point near the anterior tip could be noticed (fig. 113, right). The nuclei, which are often of unequal form and size, are placed side by side so closely that it is often difficult to distinguish them.

When the spores are fixed with Schaudinn's, Carnoy's or sublimate-acetic mixture and subjected to Feulgen's nucleal reaction, two closely associated nuclei become distinctly visible near the middle of the spore, although other structures are hardly noticeable (figs. 111, 112). From these observations, it is considered that the binucleate sporoplasm is a girdle located near the middle of the spore.

When the spores are subjected to mechanical pressure or treated with hydrogen peroxide, the polar filament is extruded from the narrower anterior end (figs. 115, 116, 165-170). The process of extrusion is similar to that observed before in other species (Kudo, 1913, 1918, 1925a). Fully extruded filaments measure 45 to 62 $\mu$ in length. Frequently the filaments show some 15 to 20 undulations of nearly the same height, but soon they become straightened, though the distal portion may exhibit some 8 to 10 undulations, as was observed in *Nosema apis* (Kudo, 1921). Incompletely extruded filaments usually show a small knob at the distal end, as previously observed in many species (Kudo, 1925a).

As to the function of the polar filament of the microsporidian spore, I have summarized before the information available up to 1924 (Kudo, 1924a). The only additional paper which has appeared since that time is that of Ohshima (1937) on *Nosema bombycis.* Ohshima maintains that the binucleate sporoplasm leaves the spore through the extruded filament and "is directly injected by the filament into the tissue." This author failed to take into account the fact that the filament when extruded measures 57-72 $\mu$ in length (Kudo, 1913) and that it is an extremely fine structure, with an estimated diameter of 0.1 $\mu$. Stages indicating the supposed passage of the sporoplasm as amoebula through this "tubular filament" are not described, nor does Ohshima consider the force with which the amoebula is "injected" through the filament without injury. On the other hand, actual emergence of the amoebula from the foramen of the spore has been observed in a number of species by different investigators; for example, *Nosema apis* (Trappmann, 1923, 1926), *Stempellia magna* (Kudo, 1924a, 1925a), *Plistophora blochmanni* (Zwölfer, 1926). Therefore, the hypothesis of Ohshima does not seem to hold true.

The characteristically resistant membrane of the microsporidian spore has been found to be of a single piece in the majority of known species. Several investigators claim to have seen two valve-cell nuclei in the developing sporoblasts. In *Nosema bombycis,* Stempell (1909) described two such nuclei which other workers who studied the same species did not observe. Fantham and Porter (1912) figure similar nuclei in *Nosema*

*apis.* In *Pyrotheca incurvata* and *Cougourdella magna,* Hesse (1935) figures two "parietal nuclei," although the spore membrane of the mature spores is a single piece. In certain species, the spore possesses a distinctly visible longitudinal line, which appears to be the sutural line of two valves of the membrane. In *Thelohania opacita,* I have observed splitting of the two valves along this line (Kudo, 1924a, 1925). In *Glugea anomala, Thelohania giardi* (Thélohan, 1895), *Plistophora simulii* (Lutz and Splendore, 1904) and *Plistophora* sp. (Mercier, 1908), a sutural line was noticed on the spores.

From the observations described above, it is concluded that the spore of *Nosema notabilis* is composed of a single-piece membrane, the binucleate sporoplasm is a girdle-shaped ring, and the spirally-coiled polar filament is located in the space unoccupied by the sporoplasm. Whether the polar filament is enclosed within a polar capsule, as in the case of *N. bombycis* (Kudo, 1916), cannot be determined.

With respect to the structure of the microsporidian spore, there have appeared a large number of papers. Some of the differences in interpretation are due to the minuteness of the object and the technical difficulties in demonstrating the inner structure. Furthermore, there has been a tendency among many authors to generalize specific information on one or a few species over the entire group of Microsporidia. Since my previous summaries on the structure of microsporidian spores (Kudo, 1920, 1924a, 1930, 1930a), very little knowledge has been added, nor does the present study contribute much to the information on this subject. The question will undoubtedly continue to be discussed so long as the present limitation in optical apparatus exists.

Based on observations on many species of Microsporidia belonging to different genera, I maintain the previously expressed view that "structural variations exist among the spores of different species." These variations may be conveniently grouped under the following types:

(1) The spore is ovoidal and contains two polar capsules, one at each end of the spore. The sporoplasm is located between the polar capsules. Thus it is comparable with various Myxosporidia in the family Myxidiidae (Kudo, 1920a). The sole species of this type, *Telomyxa glugeiformis,* has been seen only by Léger and Hesse (1910) in the adipose tissue of the larvae of *Ephemera vulgata* in France.

(2) The spore is cylindrical, with or without a caudal prolongation. Polar filament is composed of rod-like portion and a filament. According to Léger and Hesse (1916), the sporoplasm is located at the posterior end of the spore, but according to Jírovec (1936, 1936a), the sporoplasm, with one or two elongate nuclei, surrounds the rod, and is not at the posterior end. Examples: *Mrazekia caudata* (Léger and Hesse, 1916), *Bacillidium argoisi* (Léger and Hesse, 1916; Jírovec, 1936).

(3) The spore is pyriform. The polar capsule is conspicuous and median or lateral in its position, occupying the anterior two-thirds or nearly the entire length of the spore. The sporoplasm is located in the posterior region of the spore cavity, but may extend toward the middle. Examples: *Plistophora macrospora* (Léger and Hesse, 1916), *Stempellia magna* (Kudo, 1921a, 1925a), *Glugea danilewskyi* (Guyénot and Naville, 1922), *Pyrotheca incurvata* (Hesse, 1935). In *Nosema aedis,* I have noticed, in addition to the typical spore, various spores in which the sporoplasm was not terminal, but located near the middle as a complete or broken ring (Kudo, 1930).

(4) The spore is ovoidal or ellipsoidal. The sporoplasm is a girdle-like ring, located near the middle, surrounding the spirally coiled polar filament. The polar capsule may or may not be present. The great majority of the known Microsporidia possess spores which come under this group. The present microsporidian is an example. Some others in which the polar capsule was reported to be present are *Nosema bombycis* (Stempell, 1909; Kudo, 1916), *Thelohania giardi* (Mercier, 1909), and *Nosema nonagriae* (Schwarz, 1929). Those without the polar capsule are *Plistophora longifilis* (Schuberg, 1910), *Glugea anomala, G. hertwigi* (Weissenberg, 1913), *Plistophora blochmanni* (Zwölfer, 1926), and *P. simulii* (Debaisieux, 1928).

Common to all microsporidian spores is the polar filament. It has been seen coiled within the immature spores of several species, as for example, *Stempellia magna, Nosema aedis,* etc., and has, as far as my observation goes, been demonstrated to extrude under experimental conditions. Indeed, the polar filament is the most important characteristic for the identification of the microsporidian spores.

As was pointed out above, the sporoplasm varies in its position from median to terminal, and in form, from a complete or incomplete ring to a spheroidal mass. As to the number of nuclei present in the sporoplasm of the spore, there are diverse observations. The tetranucleate condition of the sporoplasm first reported by Stempell (1904, 1909, 1919) in *Glugea anomala, Nosema bombycis,* and *N. marionis,* and by Mercier (1908) in *Thelohania giardi,* has not been observed by recent workers. And in the case of *Nosema bombycis,* all other investigators found only one or two nuclei in the sporoplasm. I have recently found two sporoplasm nuclei in this microsporidian by employing Feulgen's nucleal reaction, thus confirming my previous observation with nuclear stains (Kudo, 1916) and also the findings of Ohshima (1937).

A single nucleus was observed by Schuberg (1910) in the sporoplasm of *Plistophora longifilis.* Since that time, the same condition has been reported in the following species: *Nosema aedis* (Kudo, 1930), *N. baetis* (Kudo, 1921a), *N. cyclopis* (Kudo, 1921b), *Thelohania acuta* (Schröder,

1914), *T. corethrae* (Schuberg and Rodriguez, 1915), *T. legeri, T. indica, T. obscura* (Kudo, 1929), *T. obesa* (Kudo, 1925), *T. pyriformis* (Kudo, 1924b), *T. vandeli* (Poisson, 1924), *T. varians* (Debaisieux, 1919), *Stempellia magna* (Kudo, 1924b, 1925a), *Plistophora schubergi* (Zwölfer, 1927), *Pyrotheca incurvata* and *Cougourdella magna* (Hesse, 1935).

One or two nuclei were reported in the sporoplasm of the following species: *Nosema bombycis* (Léger and Hesse, 1907; Ohmori, 1912), *Toxoglugea mercieri* (Poisson, 1924). In *Plistophora blochmanni,* Zwölfer (1926) was inclined to believe that the mature spore was uninucleate, but became binucleate when the spore entered the host's intestine. In *Nosema nonagriae,* Schwarz (1929) saw one or two nuclei, the latter being more numerous.

Two nuclei were normally observed in the following species of Microsporidia: *Nosema apis* (Fantham and Porter, 1912; Maassen, 1912; Trappmann, 1923, 1926), *N. binucleatum* (Weissenberg, 1926), *N. bombi* (Fantham and Porter, 1912), *N. bombycis* (Kudo, 1916), *N. bryozoides* (Schröder, 1914), *N. frenzelinae* (Léger and Duboscq, 1909), *N. glossiphoniae* (Schröder, 1914), *N. nepae* (Poisson, 1928), *N. notabilis, Glugea danilewskyi* (Guyénot and Naville, 1922), *Perezia legeri, P. mesnili* (Paillot, 1929), *Thelohania ephestiae* (Mattes, 1928), and *Plistophora macrospora* (Léger and Hesse, 1916).

Since recognition of the nucleus has been, and still is, dependent on certain nuclear stainings such as Heidenhain's iron haematoxylin or Giemsa's stain, it is interesting to compare the number of nuclei seen in the sporoplasm after nuclear staining and after Feulgen's nucleal reaction:

| *Microsporidia* | *Nuclear Staining* | *Nucleal Reaction* |
|---|---|---|
| *Nosema apis* | 2 (Fantham and Porter, 1912) | 2 (Jírovec, 1936a) |
| | 1 (Kudo, 1921) | |
| *N. binucleatum* | 2 (Weissenberg, 1926) | 2 (Jírovec, 1936a) |
| *N. bombycis* | 4 (Stempell, 1909) | 2 (Ohshima, 1937) |
| | 1-2 (Ohmori, 1912) | |
| | 2 (Kudo, 1916) | 2 (this paper) |
| *N. bryozoides* | 2 (Schröder, 1914) | 2 (Jírovec, 1936a) |
| *N. cyclopsis* | 1 (Kudo, 1921) | 1 (Jírovec, 1936a) |
| *N. notabilis* | 2 (this paper) | 2 (this paper) |
| *Glugea acerinae* | 1 (Jírovec, 1930) | 1 (Jírovec, 1930) |
| *G. anomala* | 4 (Stempell, 1904) | |
| | 1 (Weissenberg, 1913; Debaisieux, 1920) | 1 (Jírovec, 1932) |
| *G. hertwigi* | 1 (Weissenberg, 1913) | 1 (Jírovec, 1932) |
| *Thelohania fibrata* | 1-2 (Strickland, 1913) | 1 (Jírovec, 1936a) |
| *T. legeri* | 1 (Kudo, 1921, 1924) | 1 (Jírovec, 1932) |
| *T. mülleri* | 2 (Stempell, 1902) | 1 (Jírovec, 1936a) |
| *Bacillidium argoisi* | 2 (Léger and Hesse, 1916) | 1 (Jírovec, 1936a) |

Thus it appears that one or two nuclei are most commonly present in the sporoplasm of the majority of species of Microsporidia.

Concerning the mode of infection of *Sphaerospora polymorpha* by *Nosema notabilis,* nothing is known. As to how the trophozoites of *Ceratomyxa coris* become infected by *Nosema marionis,* Stempell (1919) conjectured that when the spores of the two cnidosporidians are ingested by the host fish, the amoebulae leave the spores in the host's intestine and Nosema amoebulae attack young trophozoites of *Ceratomyxa coris* in the gall bladder, since he did not see any Ceratomyxa spores infected by *Nosema marionis.* He noticed infected host trophozoites attached in groups to the bladder epithelium and considered that a small number of Nosema amoebulae was probably able to infect a large number of Ceratomyxa trophozoites through the plasmotomic divisions of the hosts.

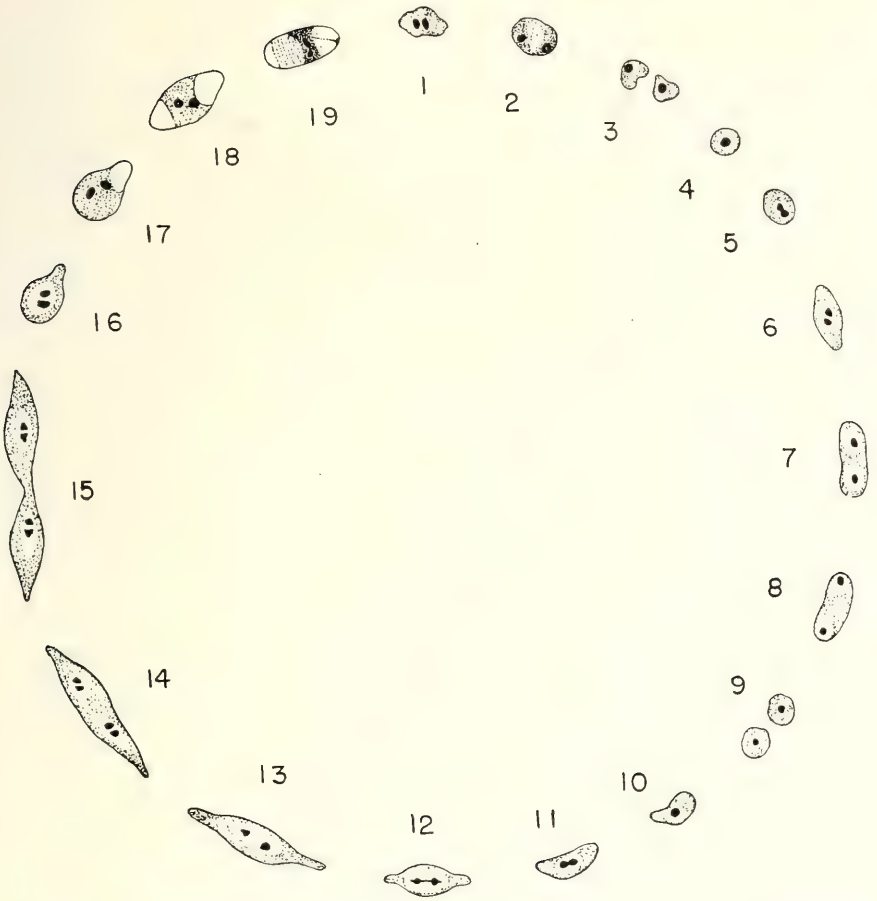
As in the case observed by Stempell, no direct infection of developing or mature Sphaerospora spores by *Nosema notabilis* has been observed up to the present time. I am therefore inclined to think that the entrance of Nosema amoebulae into the urinary bladder of the toadfish is accomplished in a way similar to that suggested for *Sphaerospora polymorpha* (p. 33), and that the microsporidian invades the host trophozoites in the fish bladder.

### Résumé of Development of *Nosema notabilis*

The development of *Nosema notabilis* as observed in the trophozoites of *Sphaerospora polymorpha* is summarized in text fig. 7. The youngest binucleate schizont (1) divides into two uninucleate schizonts (2, 3) which in turn undergo a binary fission (4-9). The schizont grows (10) and develops into a spindle-form schizont (11-13). Nuclear division (14) is followed by division into two or three binucleate spindle-form schizonts (15). A sporont (16) transforms itself directly into a sporoblast (17, 18) and develops into a spore (19). The changes between stages 19 and 1 are unknown at present.

### Taxonomic Consideration

From the observations described in the foregoing sections, it is clear that the organism under consideration is a true microsporidian, which should be placed in the genus Nosema in the present taxonomic scheme (Kudo, 1924a; Jírovec, 1936a). In 1924, I summarized information concerning the Microsporidia known up to that time, and described 53 known species of the genus. In 1936, Jírovec published a list of 16 additional species of the genus reported up to 1936. Since that time 5 more species of Nosema have been described. These are *Nosema haematobium* (Jírovec, 1936b), *N. carpocapsae* (Paillot, 1938), *N.* sp. (Kudo, 1938), *N. cactoblastis* and *N. cactorum* (Fantham, 1939). Thus at present 74 species of Nosema are on record. This number includes several incompletely studied




TEXT FIG. 7.—Diagram showing the schizogony and sporogony of *Nosema notabilis* as seen in the trophozoites of *Sphaerospora polymorpha*.

or highly ambiguous species which may not be microsporidians at all or which may belong to other genera.

The species of Nosema are widely distributed among invertebrates and lower vertebrates. While there are a few forms such as *N. bombycis* which attacks all tissue cells of all developmental stages of *Bombyx mori* in nature and which also invades under experimental conditions at least four other lepidopterous insects (Stempell, 1909; Ohshima, 1935; Kudo and De Coursey, 1940), the majority have been found to parasitize particular tissue cells of a specific host animal. In spite of the absence of our knowledge as to how specific the host-microsporidian relationship is, the difference in the host species has to be used as one of the important bases

in the identification of the microsporidian species. In *Nosema notabilis,* the association of the host and the parasite is so unique and unusual that the specific relationship between the two protozoans may be emphasized.

Comparison of *Nosema notabilis* with other recorded species in the genus shows that it differs in host association and in characteristics of the spore and developmental stages. Special attention, however, must be given to a comparison between it and *Nosema marionis,* for both are parasitic in the trophozoites of coelozoic myxosporidians in marine fishes.

*N. marionis* was first discovered by Thélohan (1895) in the gall bladder of *Coris julis* and *C. giofredi* at Marseille. Apparently, as pointed out by Stempell later, there were present no spores of a myxosporidian observed by Georgévitch (1917). Thélohan assumed the whole amoeboid organism, with an average diameter of 40 to 55 $\mu$, a microsporidian trophozoite, and named it *Glugea marionis.* Thélohan characterized the spores by stating: "en forme d'ovoide très allongé; très peu atténuées en avant; la largeur est comprise deux fois et demie dans la longueur. Longueur 8 $\mu$, largeur 3 $\mu$." Georgévitch (1917) also observed a microsporidian in the same organ of the same host fishes which he thought was identical with what Thélohan had described. Finding elongate fusiform spores in the cytosome of sporulating trophozoites of *Ceratomyxa coris,* he maintained that the two cnidosporidians underwent accidental plasmogamy, but did not state whether the two organisms lived separately and independently also. Finally, Stempell (1919), who studied the two cnidosporidians in the same two host fishes, came to the conclusion that the microsporidian was a species of Nosema and that it was parasitic in the cytosome of the myxosporidian. The spores were elongate-oval, with or without a clear rounded space at one end, and varied in length from 1.5 to 7 $\mu$. The width is not given, but the figures of the spores given by Stempell (1919: figs. 77-79) are similar to those figured by Thélohan. Stempell stated that its development was similar on the whole to that of *Nosema bombycis,* quoted before.

In being a parasite in myxosporidians, *Ceratomyxa coris* and *Sphaerospora polymorpha,* respectively, *Nosema marionis* (Thélohan) and the present species are alike, but resemblances stop there, for the chief characteristics of the spores and schizogonic changes differ too greatly between them. Moreover, *Ceratomyxa coris* has been an exclusive inhabitant of the gall bladder of two species of Coris in European waters, while *Sphaerospora polymorpha* inhabits the urinary bladder of the toadfish in North American waters. Therefore, the microsporidian described in detail here was considered a new species and was named *Nosema notabilis* in 1939.

DIAGNOSIS OF *Nosema notabilis* KUDO

*Host.*—Trophozoites of *Sphaerospora polymorpha* Davis, inhabiting the urinary bladder of *Opsanus tau* and *O. beta.*

*Locality.*—Chesapeake Bay, Solomons Island, Maryland; Lemon Bay, Englewood, Florida.

*Trophozoite.*—Young forms (1.5-2 $\mu$ in diameter) binucleate, amoeboid; binary fission results in production of two uninucleate schizonts which grow and in turn undergo binary fission. Schizonts develop into binucleate spindle-form schizonts which divide. Divisions are seemingly repeated. A binucleate sporont transforms itself into a sporoblast, and this in turn develops into a binucleate spore.

*Spore.*—Ovoid to ellipsoid, with unequally rounded ends; with or without a rounded space, or "vacuole," at the more rounded extremity. Spore membrane is of one piece. Binucleate sporoplasm is a girdle-form ring. Polar filament is attached at a point near the tip of the narrower end and spirally coiled in the intrasporal space, the coil not reaching the posterior extremity. Fresh spores measure 2.9-4 $\mu$ long by 1.4-2.5 $\mu$ wide. The smallest spores were 2.9 by 1.4 $\mu$; the largest, 4.8 by 2 $\mu$. When extruded, the polar filament reaches a length of 45-62 $\mu$.

*Remarks.*—When lightly infected, the spore formation in the host trophozoites proceeds more or less normally; when infection is severe, the host nuclei undergo hypertrophy and degeneration, and no spore formation takes place. The microsporidian is therefore considered as a pathogenic parasite of the myxosporidian.

## VI. SUMMARY

(1) Eighty-two toadfish (*Opsanus tau* and *O. beta*) obtained from Maryland and Florida were all found to harbor in their urinary bladders a coelozoic myxosporidian, *Sphaerospora polymorpha* Davis, which in turn was infected by a microsporidian, *Nosema notabilis* Kudo.

(2) The infection of *Sphaerospora polymorpha* by *Nosema notabilis* is the fifth case of hyperparasitism known up to the present time, in which a parasitic protozoan is infected by a microsporidian.

(3) The urinary system of *Opsanus tau* and *O. beta* shows a distinct sexual dimorphism. In the male, the urinary bladder has two horns, the left being larger than the right, while in the female there is only the left horn.

(4) *Sphaerospora polymorpha* was found exclusively in the urinary bladder. The trophozoites are mostly attached to the bladder epithelium in scattered groups or in one to several layers. The cytosome of the

bladder epithelial cells is destroyed by the parasites, but the host nuclei are little affected.

(5) The trophozoites multiply by a simple and possibly multiple plasmotomy. The generative nuclei divide by mitosis, in which four chromosomes become apparent, while the vegetative nuclei multiply by amitosis.

(6) The generative nuclei undergo meiotic division in which the two daughter nuclei receive two chromosomes each. Two such cells unite and form a sporont (or pansporoblast), but the two nuclei remain separate. The smaller nucleus divides once, while the larger nucleus multiplies repeatedly until twelve nuclei, all haploid, are formed. The sporont now differentiates into two sporoblasts, which in turn develop into two spores. The two haploid sporoplasm nuclei fuse into a single diploid nucleus before further development takes place.

(7) *Sphaerospora polymorpha* is compared with other known species of the genus and a diagnosis of the species is given.

(8) *Nosema notabilis* is an exclusive parasite of *Sphaerospora polymorpha.* In severely infected host myxosporidians, the generative nuclei are hypertrophied and degenerated, and no spore-formation takes place. *Nosema notabilis* is a true parasite of *Sphaerospora polymorpha.*

(9) Schizogony is by binary fission. No sexual process has been observed in the development of *Nosema notabilis.* The spore possesses a binucleate sporoplasm, and the spore membrane is of a single piece. A modification of previously reported methods for extrusion and observation of the polar filament is described.

(10) *Nosema notabilis* is compared with the known species of Nosema and its diagnosis is given.

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## EXPLANATION OF PLATES

The drawings were made by means of Abbe's drawing apparatus. The photomicrographs are untouched enlargements of negatives obtained with a Phoku camera. The abbreviations used in the descriptions of figures are as follows:

B. . . . . Bouin fixation.
C. . . . . Carnoy fixation.
F. . . . . Feulgen's nucleal reaction.
G. . . . . Giemsa staining.
H . . . . . Heidenhain staining.
M . . . . . Methyl alcohol fixation.
S. . . . . Schaudinn fixation.
Sec. . . . . Serial section preparation.
Sm . . . . . Smear preparation.

## PLATE II

*Sphaerōspora polymorpha;* × 2100.

FIG. 20.—Uninucleate trophozoite. Sm.S.G.
FIGS. 21-22.—Small trophozoites with a generative and a vegetative nucleus. Sm.S.G.
FIG. 23.—Binucleate trophozoite in which the generative nucleus is undergoing division (prophase). Sm.S.G.
FIG. 24.—Trinucleate trophozoite, with one vegetative and two generative nuclei. Sm.S.G.
FIG. 25.—Similar trophozoite. Sm.B.H.
FIG. 26.—Tetranucleate trophozoite; one of the two vegetative nuclei is dividing by amitosis. Sm.S.G.
FIG. 27.—Trophozoite with 2 generative and 4 vegetative nuclei. Sm.S.G.
FIG. 28.—Trophozoite with 4 generative nuclei and a vegetative nucleus. One of the generative nuclei is dividing (metaphase). The cytoplasm contains a foreign body of homogeneous substance. Sm.S.G.
FIG. 29.—Multinucleate trophozoite with one dividing generative nucleus at anaphase and with 3 engulfed foreign bodies. Sm.S.F.
FIGS. 30-31.—Upper and lower level views of a trophozoite in which two spores are nearly mature. Sm.S.G.
FIG. 32.—Generative nucleus in early prophase. Sec.S.F.
FIG. 33.—Prophase. Sec.C.H.
FIGS. 34-35.—Prophase. Sec.S.F.
FIG. 36.—Metaphase in polar view (?). Sec.S.F.
FIGS. 37-39.—Metaphase. Sec.C.H.
FIG. 40.—Anaphase. Sec.C.F.
FIGS. 41-42.—Anaphase. Sec.C.H.
FIG. 43.—Telophase. Sec.C.H.
FIGS. 44-45.—Telophase. Sec.S.F.

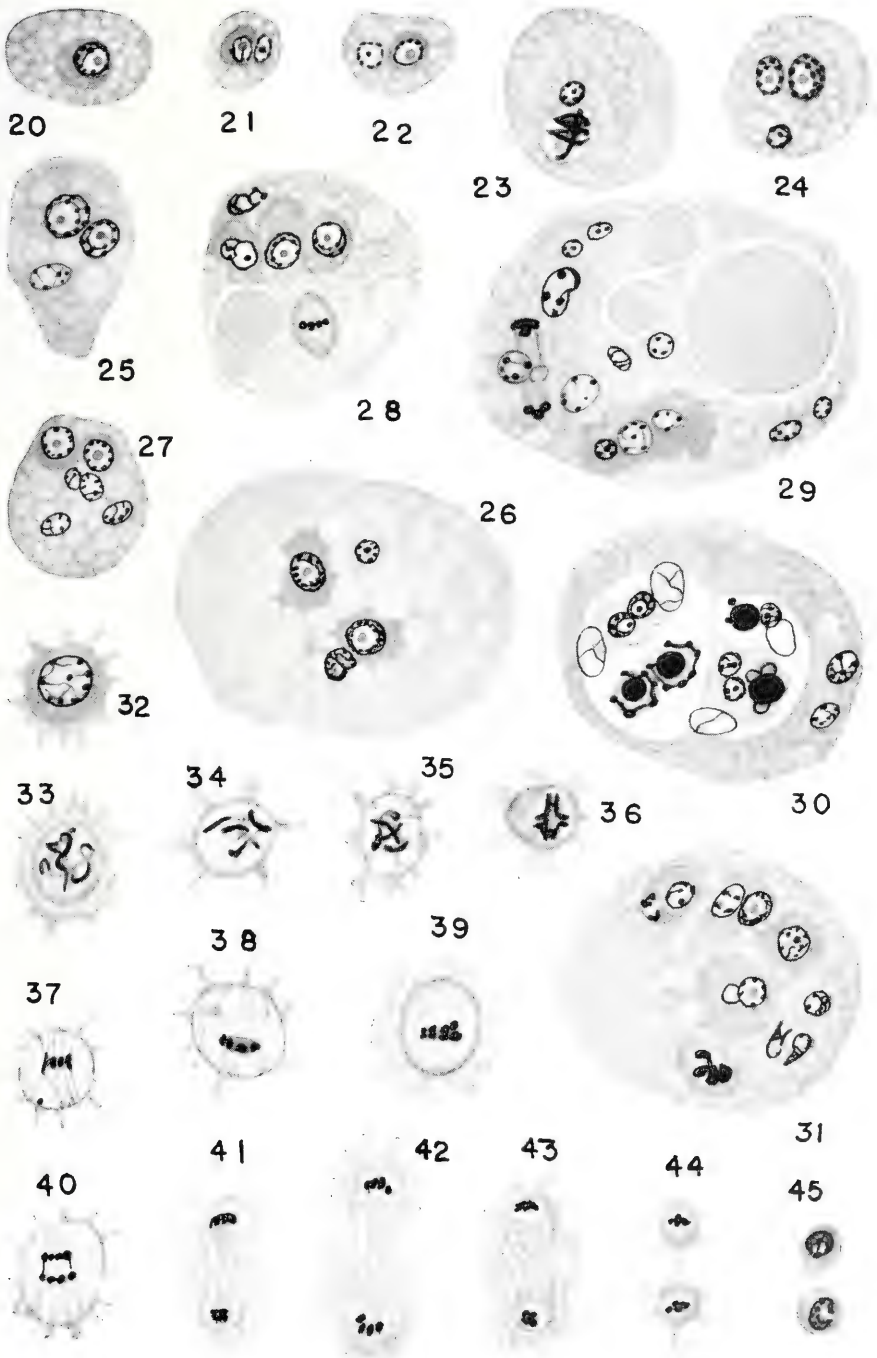
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PLATE II

## PLATE II

*Sphaerospora polymorpha;* × 2100.

FIG. 20.—Uninucleate trophozoite. Sm.S.G.
FIGS. 21-22.—Small trophozoites with a generative and a vegetative nucleus. Sm.S.G.
FIG. 23.—Binucleate trophozoite in which the generative nucleus is undergoing division (prophase). Sm.S.G.
FIG. 24.—Trinucleate trophozoite, with one vegetative and two generative nuclei. Sm.S.G.
FIG. 25.—Similar trophozoite. Sm.B.H.
FIG. 26.—Tetranucleate trophozoite; one of the two vegetative nuclei is dividing by amitosis. Sm.S.G.
FIG. 27.—Trophozoite with 2 generative and 4 vegetative nuclei. Sm.S.G.
FIG. 28.—Trophozoite with 4 generative nuclei and a vegetative nucleus. One of the generative nuclei is dividing (metaphase). The cytoplasm contains a foreign body of homogeneous substance. Sm.S.G.
FIG. 29.—Multinucleate trophozoite with one dividing generative nucleus at anaphase and with 3 engulfed foreign bodies. Sm.S.F.
FIGS. 30-31.—Upper and lower level views of a trophozoite in which two spores are nearly mature. Sm.S.G.
FIG. 32.—Generative nucleus in early prophase. Sec.S.F.
FIG. 33.—Prophase. Sec.C.H.
FIGS. 34-35.—Prophase. Sec.S.F.
FIG. 36.—Metaphase in polar view (?). Sec.S.F.
FIGS. 37-39.—Metaphase. Sec.C.H.
FIG. 40.—Anaphase. Sec.C.F.
FIGS. 41-42.—Anaphase. Sec.C.H.
FIG. 43.—Telophase. Sec.C.H.
FIGS. 44-45.—Telophase. Sec.S.F.

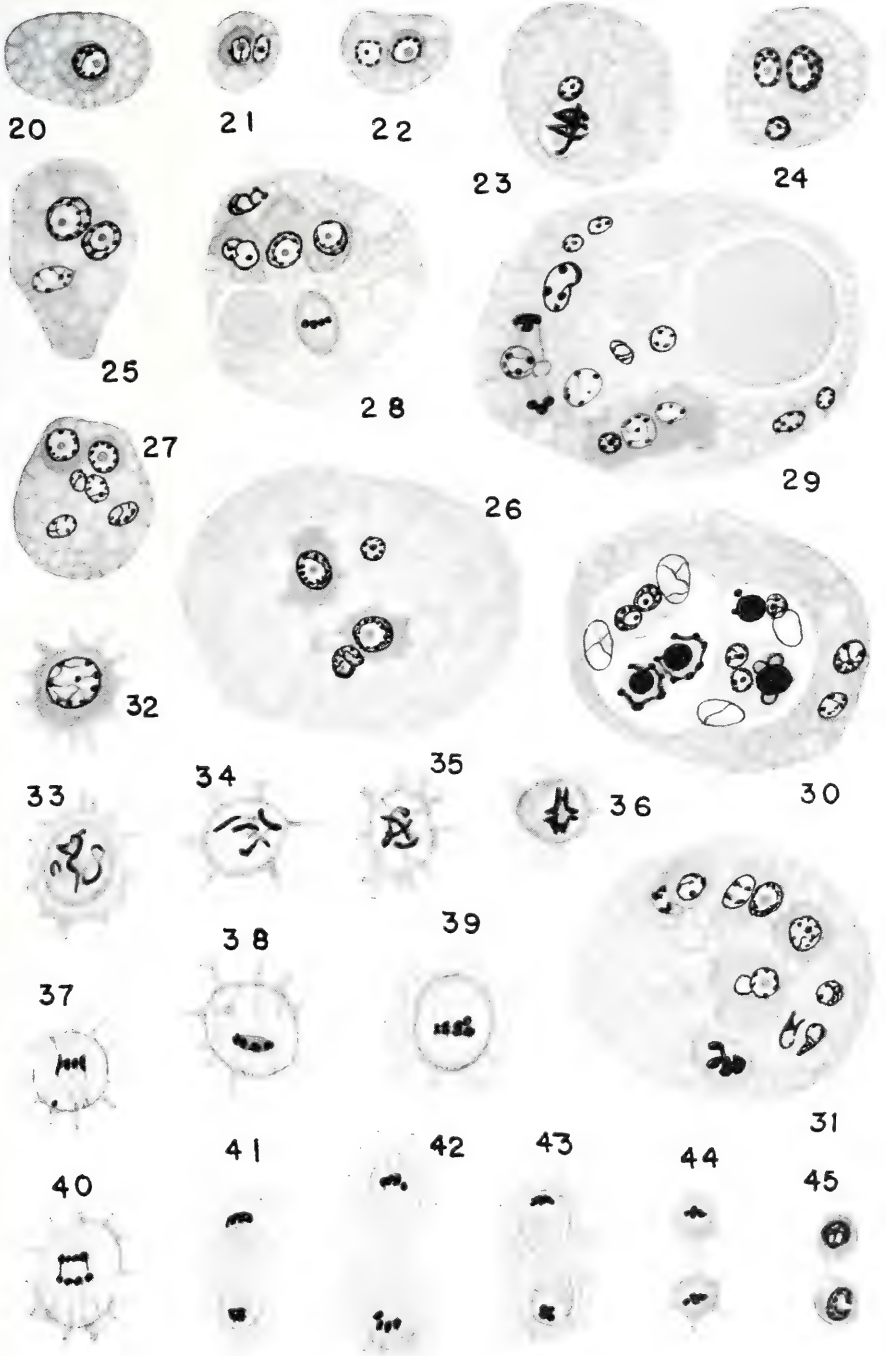
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PLATE II

PLATE III

*Sphaerospora polymorpha;* × 2100.

FIGS. 46-54.—Meiotic division stages of the generative nucleus:
46-47—Prophase, Sm.S.G. 52—Telophase, Sec.S.F.
48—Metaphase, Sec.S.F. 53-54—Telophase, Sm.S.G.
49-51—Anaphase, Sec.S.F.

FIGS. 55-57.—Association of two uninucleate generative cells, to form a sporont or pansporoblast: 55—Sec.C.H.; 56—Sec.S.H.; 57—Sm.B.H.

FIGS. 58-78.—Nuclear divisions and development of sporonts:
58-59—Sm.B.H. 68—Sec.C.H. 75—Sm.S.G.
60-61—Sec.C.H. 69—Sm.B.H. 76—Sec.C.H.
62—Sm.B.H. 70-71—Sec.S.F. 77-78—Sec.S.F.
63-66—Sec.C.H. 72—Sm.S.F.
67—Sm.B.H. 73-74—Sec.C.H.

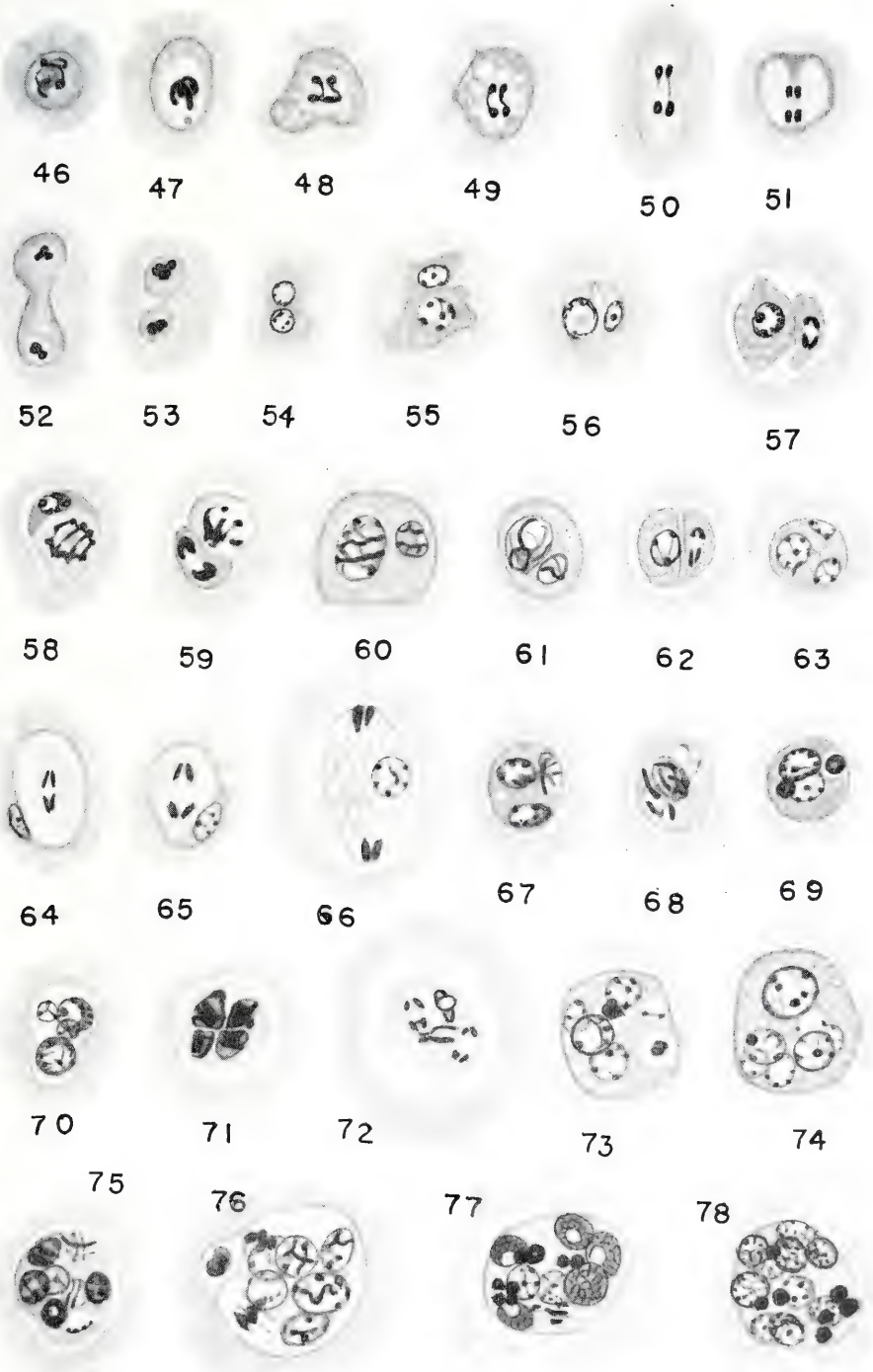
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PLATE III

## PLATE IV

*Sphaerospora polymorpha;* × 2100.

FIG. 79.—Late stage of developing sporont. Sm.S.G.
FIG. 80.—A nearly completely developed sporont, with two sporont nuclei and two young spores. Sm.S.F.
FIG. 81.—More mature spores. Sec.S.F.
FIG. 82.—Young spore. Sm.S.G.
FIG. 83.—Unusually large nearly mature spore. Sm.S.F.
FIGS. 84-85.—Two views of two almost fully formed spores. Sec.S.F.
FIG. 86.—A young spore from which the binucleate sporoplasm has been forced out. Sm.S.G.
FIGS. 87-88.—Surface views of two developing spores in which striae are being formed. Sm.S.G.
FIG. 89.—Mature spore. Sm.S.F.
FIG. 90.—Mature spore with a single nucleus in the sporoplasm. Sm.S.F.
FIG. 91.—Small spore in optical section. Sm.S.G.
FIG. 92.—Portion of a spore which has been subjected to mechanical pressure, showing the striae on the membrane. Sm.S.G.
FIG. 93.—Spore which was kept air-dried for two weeks and which extruded one of the filaments under the influence of potassium hydrate. Sm.S.G.
FIGS. 94-95.—Optical section views through the polar capsules of two fresh spores as seen in the host's urine.
FIG. 96.—Slightly oblique front view of a fresh spore.
FIGS. 97-98.—Anterior end views of two fresh spores.
FIG. 99.—Lateral surface view of a fresh spore.
FIGS. 100-104.—Abnormal spores in life.

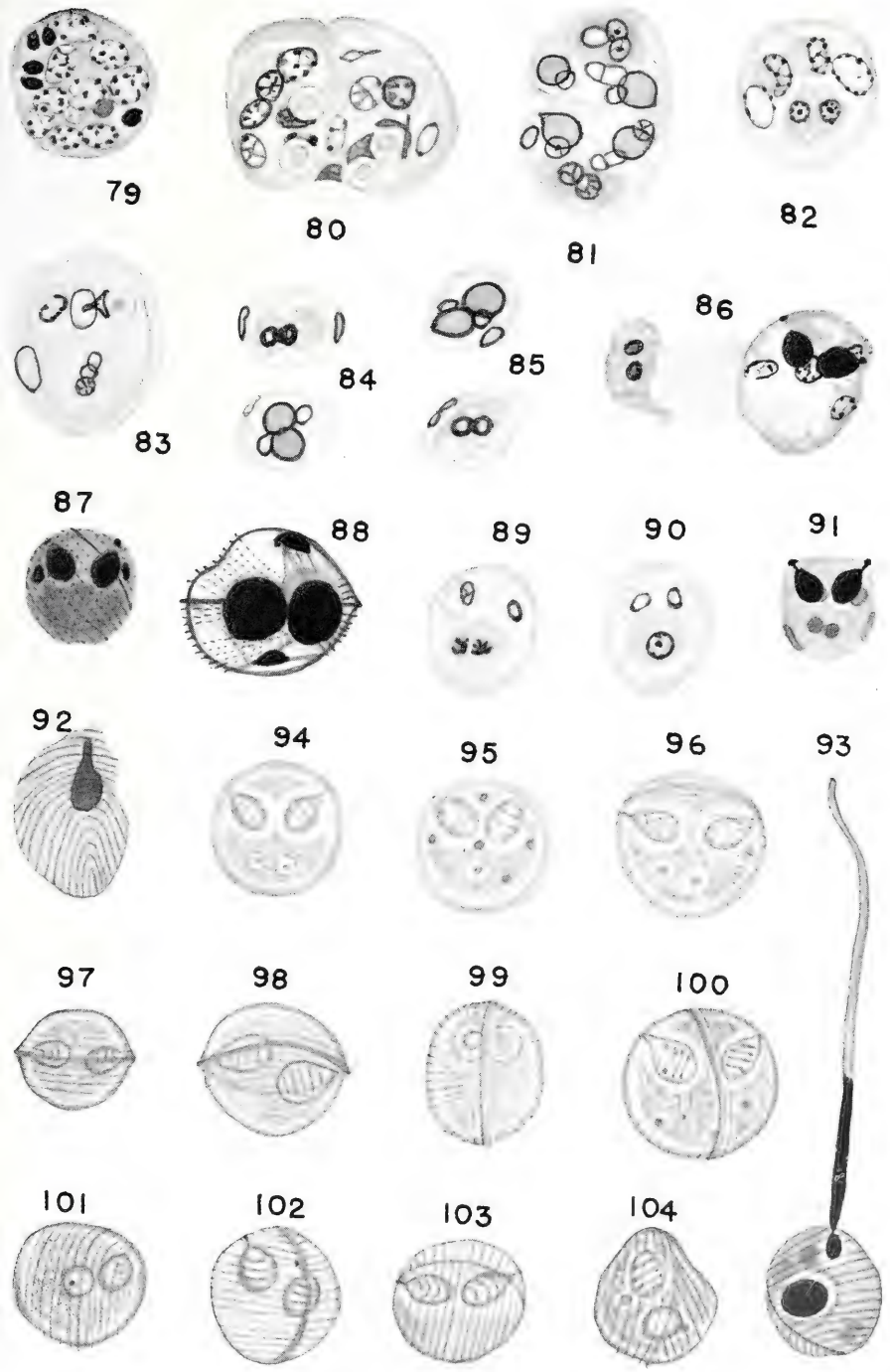
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PLATE IV

## PLATE V

*Nosema notabilis*; × 2100.

FIG. 105.—Eight fresh spores, showing different forms and dimensions.
FIG. 106.—Two different views of a single fresh spore.
FIG. 107.—Four pyriform fresh spores.
FIG. 108.—Two spores stained deeply. Sm.S.H.
FIG. 109.—A spore more decolorized. Sm.S.H.
FIG. 110.—An optical section of a similarly treated spore. Sm.S.H.
FIG. 111.—Two mature spores. Sec.S.F.
FIG. 112.—Optical section of a spore. Sec.S.F.
FIG. 113.—Five spores found in different host fish. Sm.S.G.
FIGS. 114-115.—Two spores with partially extruded polar filaments under mechanical pressure. Sm.M.G.
FIG. 116—Young binucleate trophozoite. Sm.S.G.
FIGS. 117-120.—Stages in binary fission of binucleate schizont. Sm.S.G.
FIGS. 121-127.—Stages in binary fission of uninucleate schizonts: 121-122, Sm.S.G.; 123, Sm.S.F.; 124-125, Sec.C.H.; 126, Sec.S.F.; 127, Sec.C.H.
FIGS. 128-136.—Stages in the development of spindle-form schizonts: 128-133, Sm.C.H.; 134-136, highly spread out schizonts, Sm.S.G.
FIGS. 137-141.—Further division of spindle-form schizonts: 137-140, Sm.C.H.; 141, Sec.S.G.
FIGS. 142-144.—Stages in sporont-formation. Sm.C.H.
Figs. 145-146.—Sporoblast and young spore. Sm.S.G.

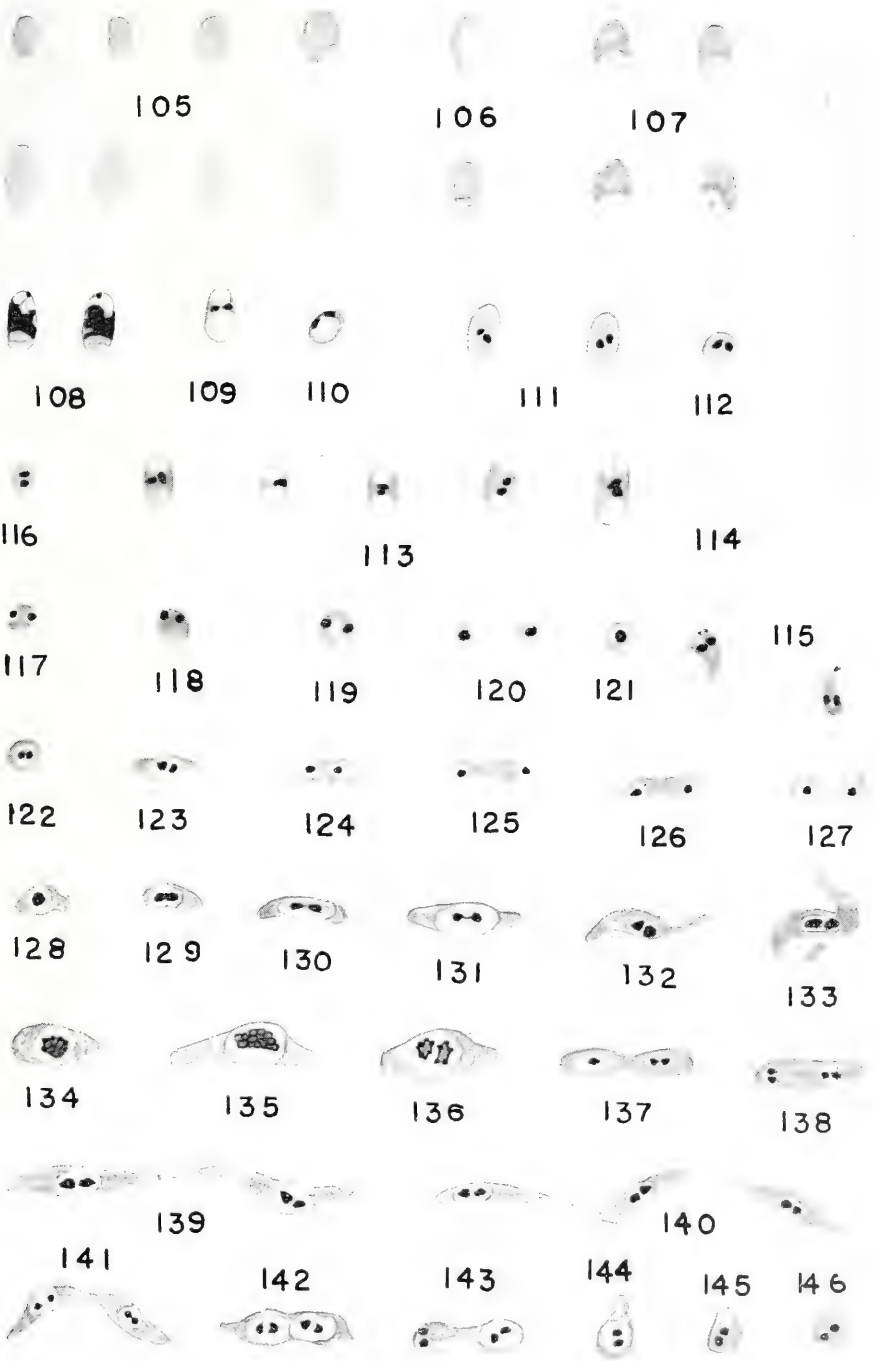
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PLATE V

PLATE VI

*Sphaerospora polymorpha* infected by *Nosema notabilis;* × 2100.

Fig. 147.—Young binucleate host trophozoite infected by five Nosema schizonts. Sm.S.G.

Fig. 148.—Tetranucleate trophozoite with eight Nosema schizonts. One host nucleus is extremely hypertrophied. Sm.S.G.

Fig. 149.—Small trinucleate trophozoite with six Nosema schizonts. Sm.S.G.

Fig. 150.—Trinucleate trophozoite infected by nine uninucleate Nosema schizonts. The three host nuclei are degenerated. Sm.S.G.

Fig. 151.—Larger trophozoite. The host nuclei are beginning to become hypertrophied. Sm.S.G.

Fig. 152.—Trophozoite lightly infected by six binucleate schizonts and a young spore of Nosema. Sm.S.G. See also fig. 171.

Fig. 153.—Another lightly infected trophozoite with thirteen Nosema schizonts. Sm.S.G. See also fig. 172.

Fig. 154.—Heavily infected trophozoite with eight somewhat hypertrophied and degenerating nuclei, filled with ten schizonts and twenty-two spores of Nosema. Sm.S.G. See also fig. 173.

Fig. 155.—Moderately infected trophozoite. Sec.S.F.

Fig. 156.—Trophozoite with twelve schizonts and two young spores of Nosema. The host nuclei appear normal. Sec.C.H.

Fig. 157.—More heavily infected host trophozoite. Sec.C.H.

Fig. 158.—Small trophozoite with nine spores and two schizonts of Nosema. Three host nuclei have degenerated. Sm.S.G.

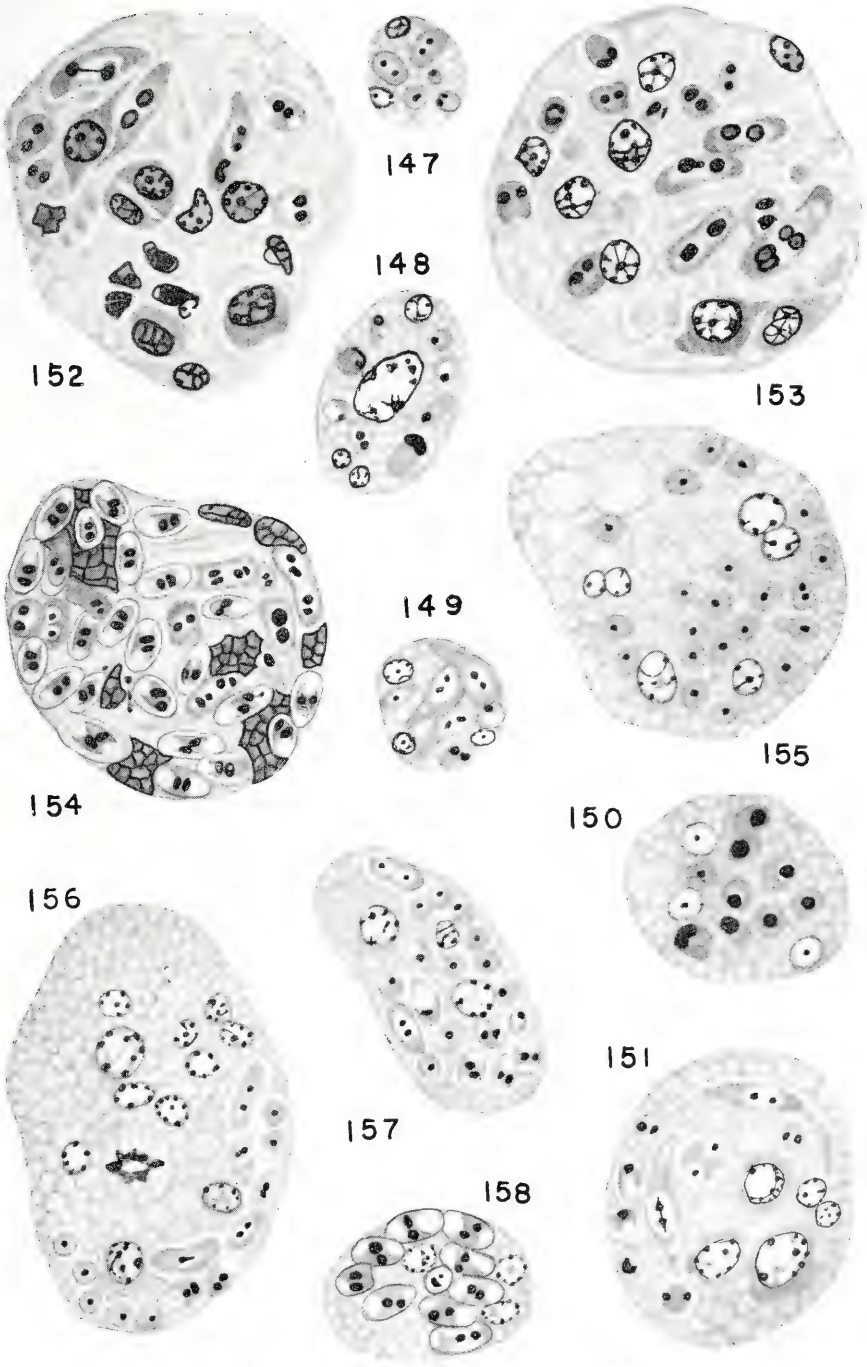



PLATE VI

## PLATE VII

FIG. 159.—Fresh scraping of the wall of the urinary bladder of a toadfish, showing trophozoites and isolated spores of *Sphaerospora polymorpha*. The microsporidian spores are indistinctly visible. Life, × 470.

FIG. 160.—Trophozoite of *Sphaerospora polymorpha*, crushed under the cover glass, showing 21 spores of *Nosema notabilis* which developed within, and at the expense of, it. Life, × 2100.

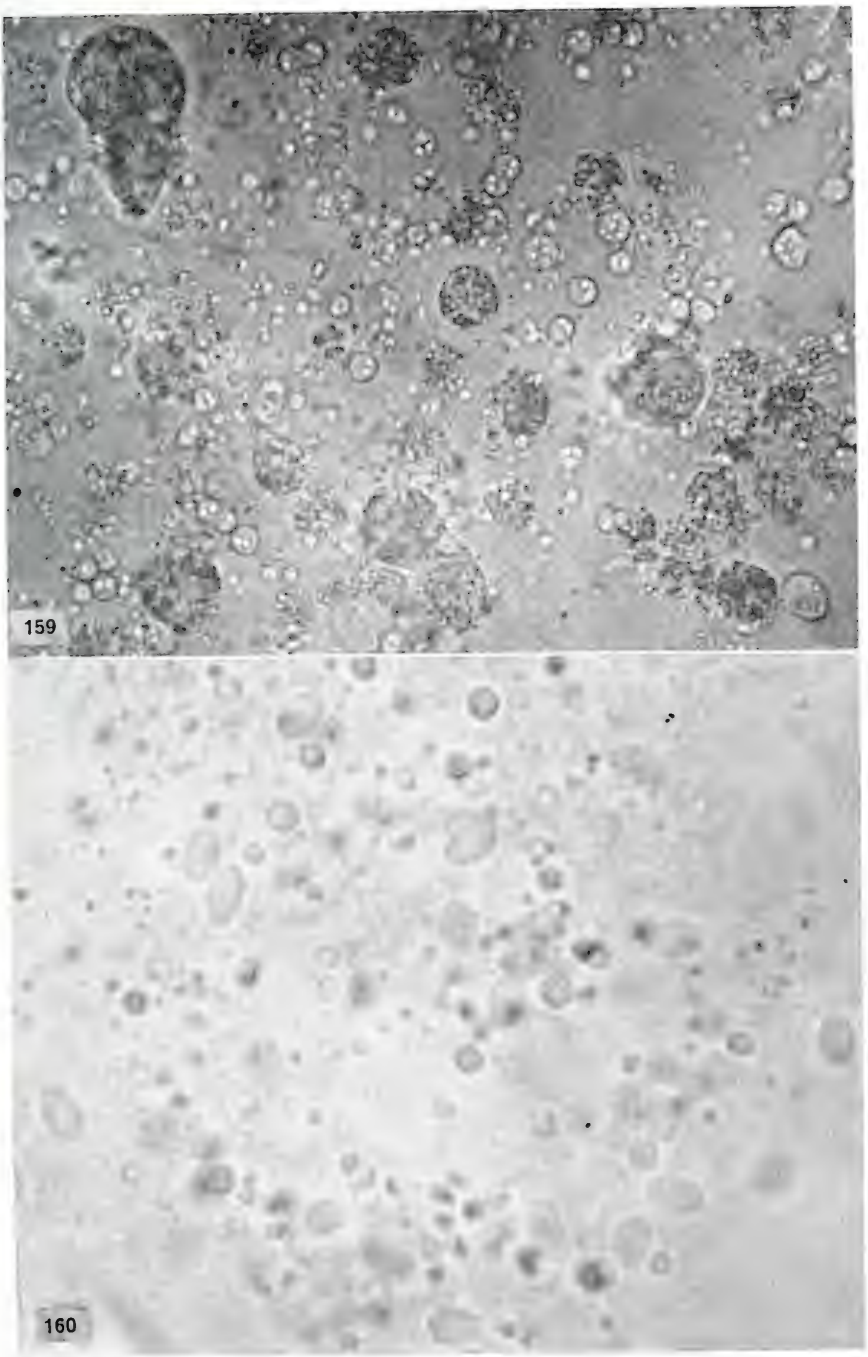
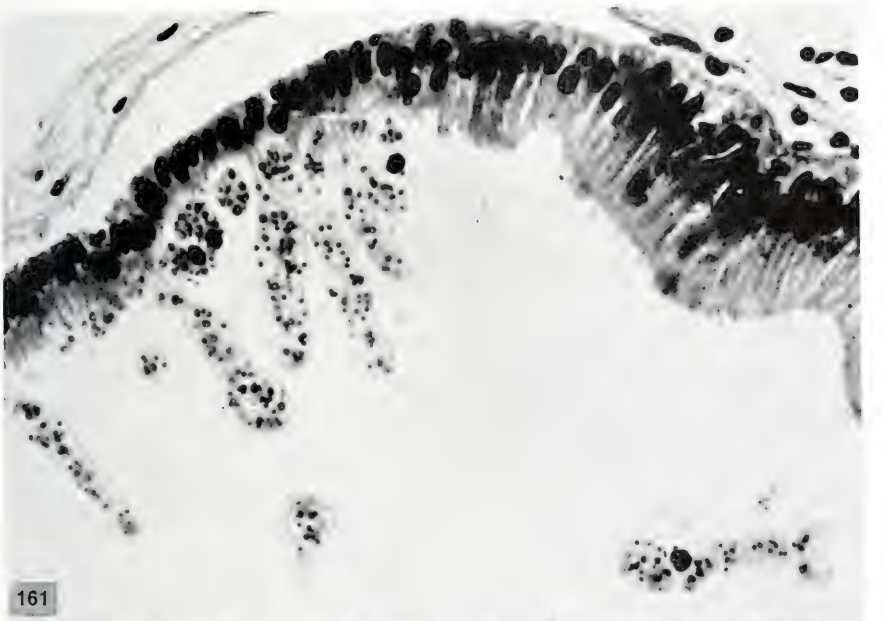



PLATE VII

PLATE VIII

FIG. 161.—Portion of the bladder epithelium of a toadfish with attached elongated trophozoites of Sphaerospora, three of which contain mature spores. Sec.S.G. × 470.

FIG. 162.—Portion of the urinary bladder of another fish, very heavily infected by *Sphaerospora polymorpha,* many of which are infected by Nosema. Sec.C.H. × 470.



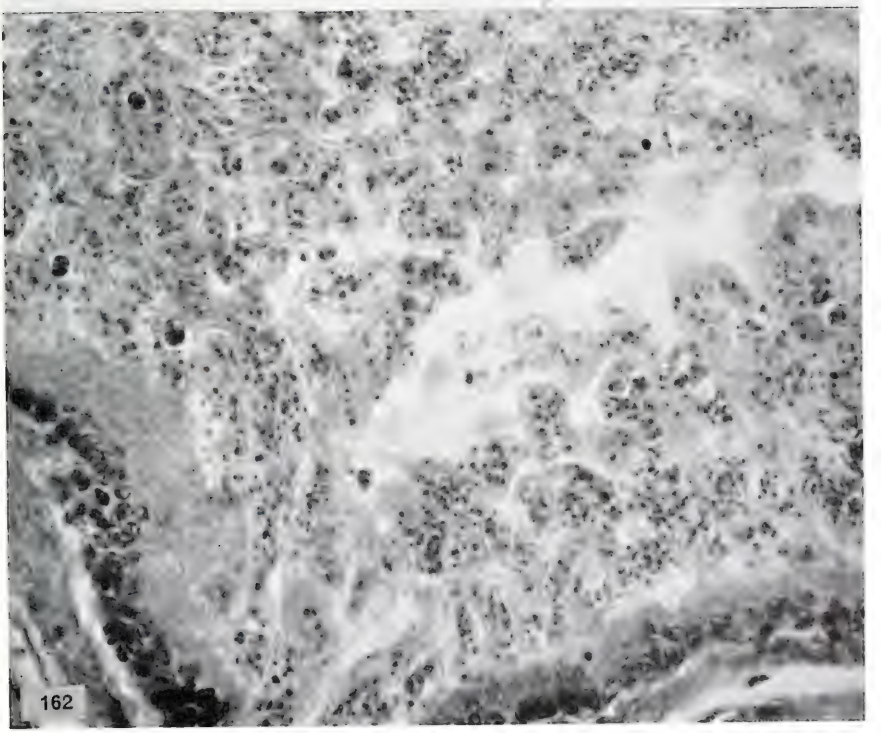




PLATE VIII

PLATE IX

FIG. 163.—Portion of the urinary bladder of a toadfish, showing eleven trophozoites of *Sphaerospora polymorpha,* three of which are heavily infected by *Nosema notabilis.* Sec.S.H. × 2100.

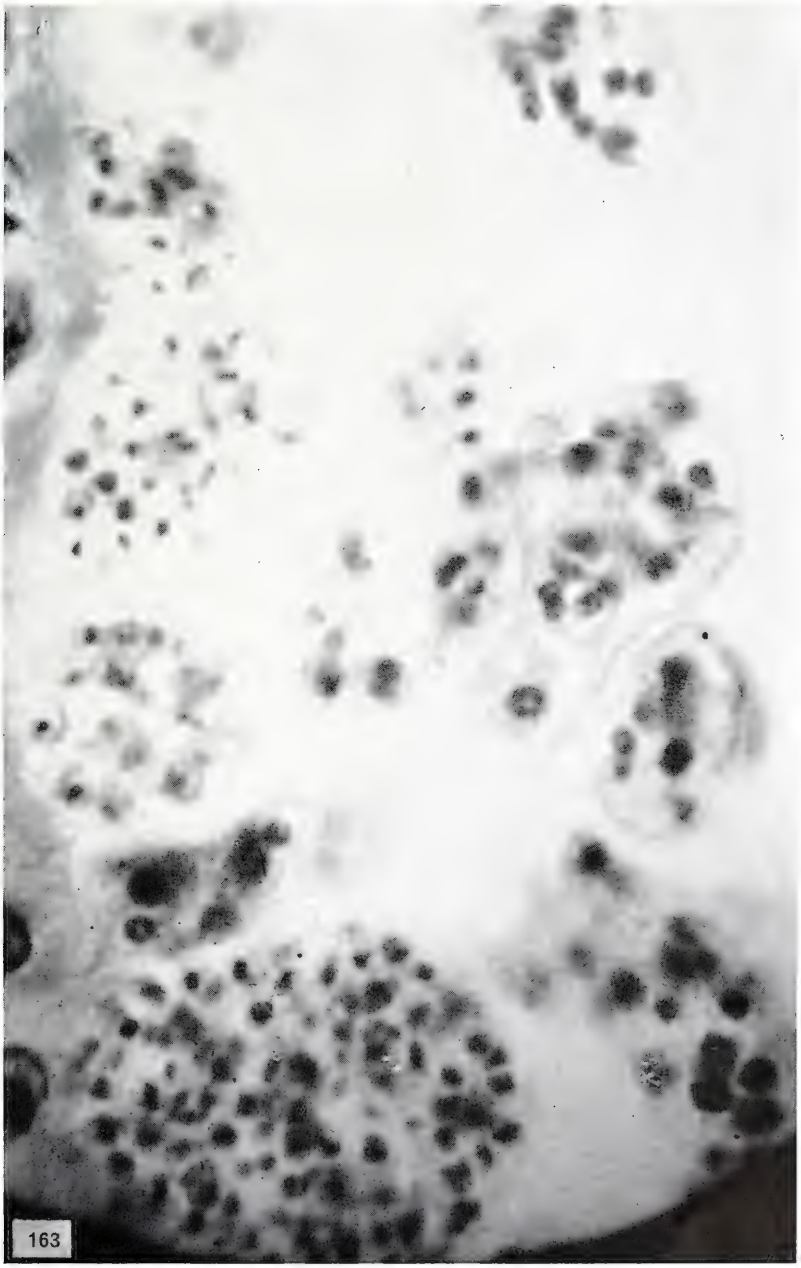



PLATE IX

PLATE X

FIG. 164.—Portion of a urinary bladder containing 12 Sphaerospora trophozoites, heavily infected by schizonts, sporonts, and spores of Nosema. Sec.S.G. × 1575.

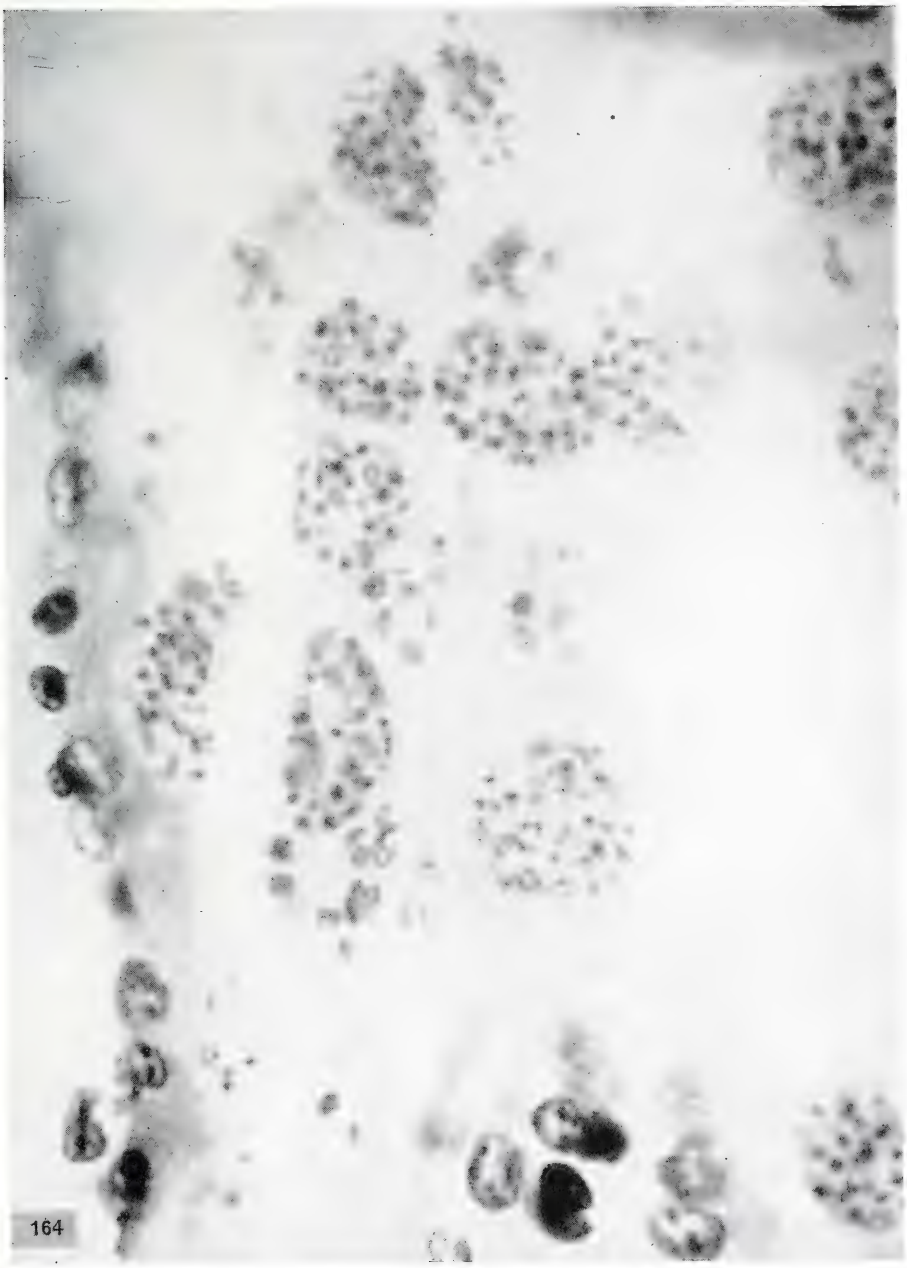



PLATE X

## PLATE XI

FIGS. 165-170.—Spores of *Nosema notabilis* with their extruded polar filaments under the influence of hydrogen peroxide or of mechanical pressure. All dark field, except fig. 167. × 1575. Figs. 166 and 167 are dark field and bright field views of the same spore. Note the faintly visible basal portion of the extruded filament in fig. 167.

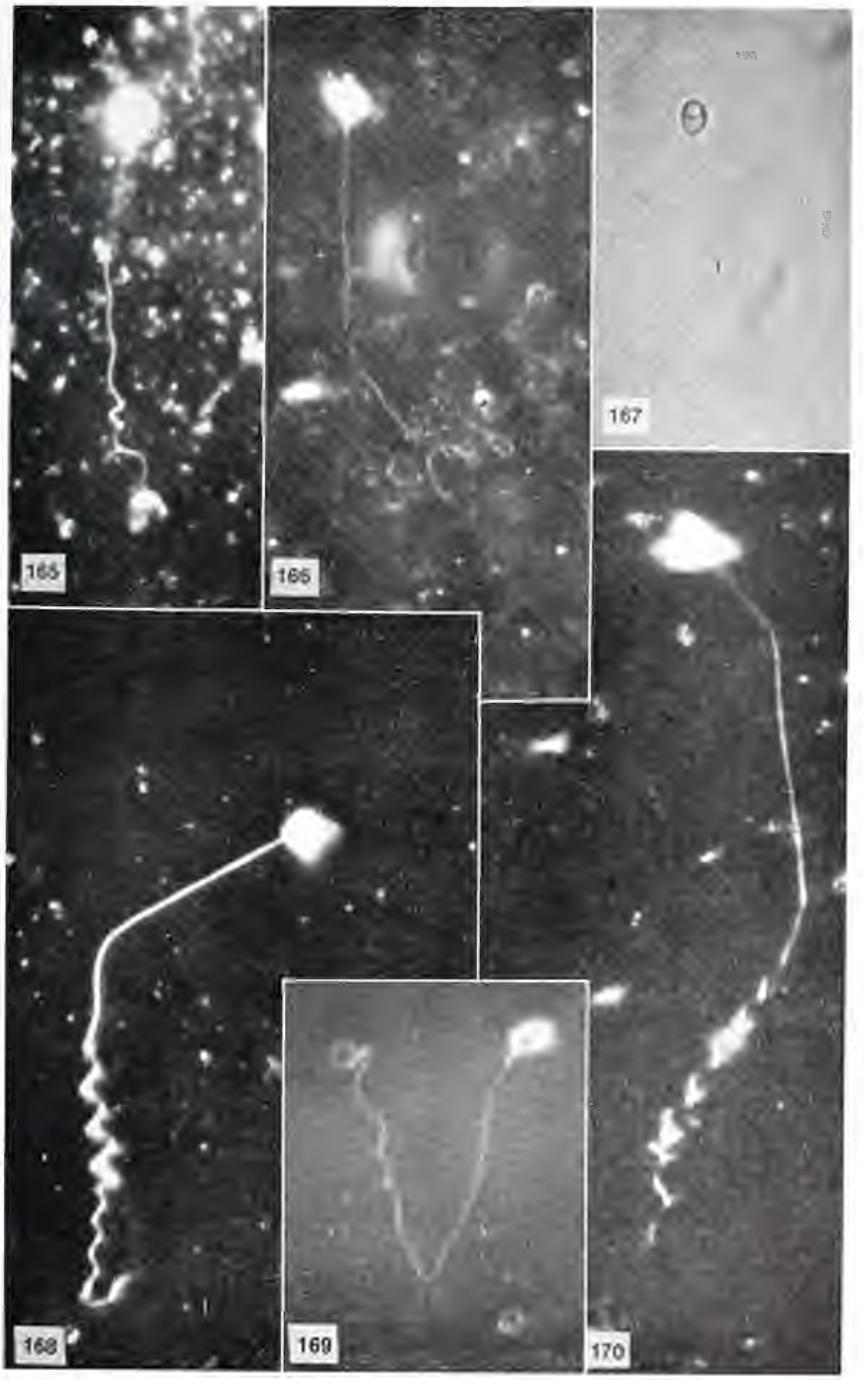
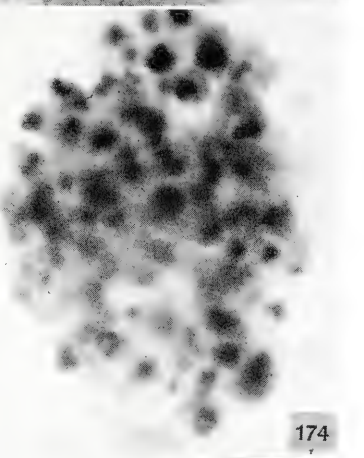



PLATE XI

## PLATE XII

FIG. 171.—Sphaerospora trophozoite shown in fig. 152. × 2050.
FIG. 172.—Sphaerospora trophozoite shown in fig. 153. × 2050.
FIG. 173.—Sphaerospora trophozoite shown in fig. 154. × 2050.
FIG. 174.—Sphaerospora trophozoite heavily infected by Nosema. Sm.S.G. × 2100.
FIG. 175.—Portion of the urinary bladder of a toadfish with Sphaerospora trophozoites which are all heavily infected by Nosema. Sec.S.G. × 1150.



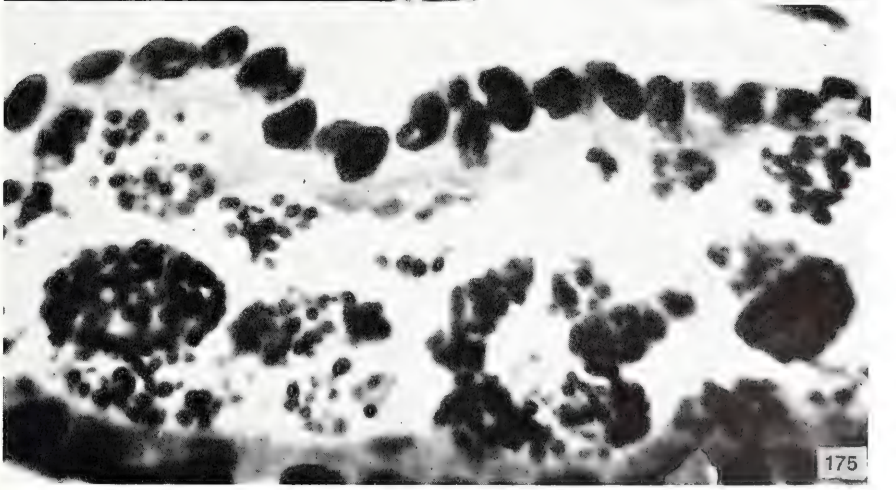




PLATE XII

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